The origin and function of odours in island birds

A thesis submitted in partial fulfilment of the requirements for the

Degree of Doctor of Philosophy in Evolution

at the University of Canterbury

Laura Azzani

2015

Abstract

The field of avian chemical ecology is very much in its infancy, but there has undoubtedly been a surge of interest in recent times for the study of odour in birds – it has even recently been termed "the New Black of avian communication". I sought to add to the field by carrying out a study on various aspects of the origin, development and function of odours in island birds.

I first explored the ontogeny of preen wax production and the development of a chemical signal in nestlings of 5 native and introduced species of New Zealand birds. I did not find significant differences in the start of preen wax production in native *vs.* introduced species, mainly due to the highly distinctive preen wax development pattern shown by the European starling (*Sturnus vulgaris*). However, I did discover that nestlings modify the composition of their preen wax over the course of the nestling phase, and the compositional change follows different trajectories in native and introduced species. Multivariate analysis of the preen wax profiles revealed significant variation at all levels examined (status, species and nest). This is consistent with the hypothesis that different evolutionary histories are reflected in the preen wax profiles of birds, and this is discernible from a very early age.

I next examined the effect of diet on the chemical profile of a generalist bird widespread in the South Pacific, the silverye *Zosterops lateralis.* Diet is often cited as a possible confounding effect in studies comparing composition of chemical signals between sexes, seasons, breeding and developmental stages, and among different species, but the effect of diet on chemical profiles has rarely been tested directly. I used a supplemental feeding experiment with tallow fat but this change in diet did not affect preen wax composition in silvereyes, and there was no change in preen wax composition among sampling periods. This supports the hypothesis that preen wax composition in silvereyes is determined by endogenous mechanisms and is not affected by day to day variation in diet.

I then explored the differences in chemical profiles of "inbred" *vs.* "hybrid" South Island robins *Petroica australis*, living sympatrically on Motuara Island, where an experimental genetic rescue translocation was carried out in 2008. This setup allowed me to investigate the influence of inbreeding and geographic origin on the chemical profiles of a native New Zealand species, without facing confounding effects derived from the birds living in different habitats. I detected a significant effect of status ("inbred" *vs.* "hybrid") on chord-transformed chemical profiles, but found no correlation between chemical profiles and heterozygosity, or between chemical and genetic distances. These results suggest that South Island robins preen wax could encode a geographic

signal rather than a heterozygosity signal.

Finally, I present the results of a broad comparative study that I undertook in collaboration with Aude Thierry, comparing chemical profiles of a number of passerine species across the South Pacific which are phylogenetically related but have very different evolutionary histories, having either evolved in Australia, under pressure from a number of olfactory-searching predators, or on islands were such predators were absent (New Zealand and New Caledonia). We found that island birds showed a significant loss of complexity in the non-volatile components but a significant increase in the diversity of compounds in the volatile part of their wax profile. This suggests birds on islands produce preen waxes, and thus odours, that are likely to be more conspicuous than those of continental species. This is concordant with our expectation, given what we know of their evolutionary history with regards to coexistence with predators, and points to the possibility that some form of “olfactory crypsis” is at work in the continental Australian birds, but is not present – or has been lost – in island birds.

Overall, my study confirms that the current interest in avian chemical communication is well warranted as there appears to be a wealth of information encoded in avian chemical signals, and we are only just starting to explore and understand the biological relevance of it, both at the intra- and inter- specific level. This could have repercussions on conservation strategies and in general practices of bird handling, and will allow us to gain an understanding of a communication system that, if ubiquitous across the animal world, has thus far been overlooked in the avian class.

Table of Contents

List of Figures

List of Tables

Acknowledgements

This PhD has been an amazing journey, full of surprises and unforeseen circumstances, right from the very start, as I applied for this post 2 days before the February earthquake hit Christchurch. Luckily, this did not prevent the project from going ahead, or me being a part of it, and I am incredibly grateful for that.

I would like to thank first and foremost my supervisor, Professor Jim Briskie, for giving me this opportunity, and for seeing me through it - never failing to believe in me and in my capacities, even when I was doubting myself, my skills, or my ability not to blow up the gas-chromatograph. He is one of the most composed and insightful people I have ever met, which comes in very handy when dealing with a temperamental Italian student!

A big thank you also goes to my co-supervisors, Steven Gieseg and Bart Kempenaers. Steven introduced me to the Free Radical Lab and gave me free rein on the Shimadzu GC, enabling me to gain a huge amount of knowledge not only on the analytical process itself, but on the optimization of the system and maintenance of the instrumentation. Bart was far away in Germany, but always available for me whenever I had a doubt, and more than willing to share his knowledge and enlist the help of others that could assist me while I found my way through the depths of analytical chemistry and signal processing. This is were I would like to extend my most sincere thanks to Andreas Reinecke at the Max Planck Institute (first Jena, then Seewiesen), who managed to “coach me” from a distance and share his insights on gas chromatography. His advice has always been extremely thorough and most welcomed, as were his words of encouragement and praise, and his constant reminding that I sure was faced with a big challenge, but I was also given a unique opportunity to gain insight and first-hand knowledge of the analytical processing of my samples, which often biologists don't get to experience.

I certainly wasn't alone in this academic journey, having been paired up with another PhD student for the project. Aude and I shared rooms, groceries, cooking duties, journal clubs, lab benches and fieldwork in three different countries, and we managed to complement each other at the professional level, giving rise to a fruitful collaboration, while getting to know each other and overcoming our differences on a personal level. Thank you for discovering with me the art of collaboration and for being a most efficient fellow PhD!

Thank you to my other fellow PhD as well, Justin Rasmussen, with whom I had the pleasure of sharing one and a half field seasons, and with whom I collaborated for one of my chapters. Doing field-work with Justin is one of my fondest memories of the PhD, and we've been helping each other out in the field and in front of the computer a lot – not to mention sharing huge plungers of

coffee at the Kaikoura field station.

Thanks to Jack Van Berkel and Ngaire Button for keeping the Edward Percival field station ship afloat during my time there, and to the University of Canterbury for providing such amazing facilities for its students and staff to use. Thank you also to all the many people who provided support for Aude and I during our trips to New Caledonia and Australia, allowing us not only to collect our samples and carry out our research, but to have a place we could call home for a few weeks at a time.

This PhD entailed considerable amounts of field work, and I would never have been able to follow enough birds or collect enough samples, had it not been for the help of my many field assistants. I would like to thank Matt Weiland, Robyn White, Lorna Deppe, Archie McFarlane, and David Lloyd-Jones. A particular thank goes to David Lloyd-Jones for being so passionate and dedicated, to Sol Heber for being the best field assistant I could have hoped for on Motuara Island, given her superior knowledge of the area and almost personal acquaintance with every single robin there, and to Jack Coggins for being my friend, my field assistant and my partner. What started as a brief vacation from your own PhD, to come robin-hunting with Sol and I, turned into a three year relationship. I for one am very grateful for that, as I'm sure are the Motuara Island robins, who for 12 days were fed mealworms aplenty and greeted by a heavily-British-accented “Hallo robin!” every time you caught one.

This project would not have been possible without a grant from the Marsden Fund to my supervisors. The fund provided me with a scholarship as well as the resources to carry out the field and lab work. I thank them for their support.

I would like to thank my parents, Imer Azzani and Anna Rita Leonardi, for bearing with me during this long time away, for being content with my very scarce Skype activity, and for picking up many an old-fashioned international phone card to call me and hear my voice when my internet presence was lacking for months on end!

Finally, I would like to thank all my many friends and acquaintances here in Christchurch, who have welcomed me with open arms and a quizzical smile since day one. “What are you doing here?”, and “Why Christchurch?” were some of the most common questions I have had throughout the years, especially in 2012, when so many people were leaving the city, and my local friends were almost moved that I had chosen to come instead. Living in Christchurch during this time hasn't always been easy, but the warmth and openness of the local community made it all definitely worth it. A special mention also goes to the international contingent: Benjamin, Camille and Maud, my lovely French speaking friends, and Rachel Harley, who is the funniest, toughest American I have ever met.

Chapter 1

General introduction

Chemical communication is ubiquitous in the animal kingdom, as decades, if not centuries, of research testify. Responses to odours emitted by a conspecific animal were documented even by Aristotle, and accounts of the phenomenon continued throughout the ages (Wyatt 2003), but the first structured studies of animal olfactory communication focused on insects and their "pheromones": these, as defined by Peter Karlson and Martin Luscher in the 1950s, are small, volatile molecules (a pheromone is one single compound, or a mixture of compounds in very precise, fixed proportions) involved solely in intra-specific communication, that elicit a fixed, innate reaction on the receiver. This definition worked well for the organisms and molecules that people were working on at the time (mainly insects, for instance the silk moth *Bombyx mori* and its sex pheromone bombykol), but it proved quite restrictive – and even controversial – for research to come.

By the 1970s, the role and functioning of mammalian chemical signals came into focus, and many of these did not seem to fit within the pheromone paradigm (e.g., Caro & Balthazart 2010). As a result, the original pheromone concept developed in studies of insects has been debated for vertebrates, and has been now superseded by a more encompassing concept of "chemical signal" or "body odour", functionally defined as "a conspecific compound(s) that affects the receiver" (Muller-Schwarze 2006). This includes a much wider range of semiochemicals, such as dominance signals, and notably, "individual signatures" that vary from individual to individual and would not have fit under the original definition of pheromone. Moreover, responses to compounds such as the *maternal pheromone* in rat faeces (Leon & Behse 1977), and phenomena such as maternal and filial imprinting, all require some degree of olfactory learning, and as such differ from the innate response with which classical pheromones are associated. Nowadays, chemoecological research has broadened its scope and most researchers use the approach illustrated by Wyatt in "Pheromones an animal behavior" (2003): "I have taken a broad and generous approach that includes many examples of important behaviours mediated or influenced by chemical cues that would currently

fall outside a rigid definition of pheromone."

Despite the recent interest in the role of semiochemicals in vertebrate communication, chemical communication in birds has not received the same attention as in other animal groups. Until recently, birds were traditionally thought to be anosmic, or microsmatic as best (Soudek 1927; Walter 1943). During the 1960s, anatomical evidence to the contrary started to accumulate, kick-started by the pioneering work of Bang describing olfactory nasal structures and detailing chemically sensitive olfactory epithelium in a range of avian species (see Bang 1960; Bang et al. 1968; Bang 1971). At the same time, physiological studies investigating electrical responses to olfactory stimuli were conducted in several species, particularly by Bernice Wenzel's lab (Wenzel 1971; Wenzel & Sieck 1972; Wenzel 2007) and others (Shumake et al. 1969; Stattelman et al. 1975). These two parallel streams of research (anatomical and electrophysiological) coalesced nicely into a broader discourse of "form and function" in the avian (olfactory) brain. Bang and Cobb's 1968 study investigated relative size of the olfactory bulb to the cerebral hemisphere, resting on the assumption that larger size corresponded to greater functionality. He found that birds in the Procellariiformes (seabirds), Apterigiiformes (kiwi) and the vulture *Cathartes aura* had the largest ratio, while Passeriformes (songbirds) had the smallest ratio. Interestingly, various electrophysiological assays with odour stimuli detected a positive response - in the form of olfactory spindles generated in the olfactory bulb (already known and recorded in mammals; Sieck & Wenzel 1969; Wenzel & Sieck 1972), and in the form of action potential from olfactory nerves (Tucker 1965). These were found in all species tested, which covered a wide range of relative olfactory bulb sizes. This pointed to the fact that all avian species possess the ability to perceive olfactory stimuli, and respond to it at least at the physiological level.

Somewhat surprisingly, given the overwhelming evidence from the anatomical and electrophysiological research, the belief that birds were anosmic, and that chemo-olfactory communication was not developed in this class, persisted. This is perhaps to do with the fact that rigorous behavioural experiments testing avian olfactory abilities were somewhat slow in following the neuroanatomical and physiological discoveries, and initially focused on those species found to have a larger olfactory bulb to brain size ratio (Stager 1964; Grubb 1973). The scarcity of early, conclusive behavioural studies confirming the role of olfaction contrasted with a number of field observations most ornithologists would subscribe to: namely, the vast majority of birds do not exhibit any overt sniffing behaviour, they do not conspicuously scent mark, and their external olfactory apparatus is rigid, with nostrils positioned dorsally, which seems ill fitted to olfactory exploration. Moreover, birds appear to devote a lot of time and energy to visual and acoustic displays, which are immediately evident and most striking to human observers, and thus these

modes of communication have monopolized the focus of avian research for decades (Bonadonna & Mardon 2013; Caro et al. 2014).

The neglect of chemical communication in birds started to change from the early 1990s, and an ever increasing number of studies focusing on the importance of olfaction in the avian world has since been published. Experiments have shown that birds use olfaction in a variety of contexts, including foraging/ food location (Graves 1992; Nevitt & Bonadonna 2005; Cunningham et al. 2009; Castro et al. 2010), predator detection (Amo e al. 2008, Roth et al. 2008; Leclaire et al. 2009), selection of nest material (Petit et al. 2002; Gwinner & Berger 2008), homing to the nest (Bonadonna & Bretagnolle 2002; Bonadonna et al. 2004) and navigation (reviewed in Gagliardo 2013). Moreover, olfaction has proven important for birds in a variety of social contexts as well, giving rise to a spate of pheromonal and socio-olfactory research across a variety of bird families. A number of reviews, in particular, have brilliantly synthesized the growing body of research in this field (Hagelin & Jones 2007; Rajchard 2007; Caro & Balthazart 2010; Campagna et al. 2012; Bonadonna & Mardon 2013; Caro et al. 2015).

With the growing realization that perception of conspecific and heterospecific odours was possible for birds, new attention has been devoted as well to the sources of such odours. Birds produce a number of odorous substances, such as faeces, stomach oils, blood, and glandular secretions (Hagelin & Jones 2007). This last group of substances, in particular, seems to hold much potential for biological communication: a number of studies have detected behavioural responses to preen wax, an oily substance produced and secreted by the uropygial gland (Whittaker et al. 2009; Zhang et al. 2010; Zhang et al. 2013). The secretion from the gland contains a large number of volatile and non-volatile compounds, and is considered to be the key source of avian body odours (Mardon et al. 2010; Campagna et al. 2012; Zhang et al. 2013; Tuttle et al. 2014).

In fact, preen wax is thought to play a number of roles beside that of olfactory signal, some of which have been confirmed via experimental evidence – antibacterial and antiparasitic functions have been demonstrated by a number of authors (Moyer et al. 2003; Shawkey et al. 2003; Burger et al. 2004; Martín-Platero et al. 2006; Reneerkens et al. 2008) - while functions such as waterproofing, maintenance of feather flexibility and protection against UV radiation have been hypothesized although not yet experimentally confirmed (Reneerkens 2007; Gunderson 2008). Given this diverse array of functionality, and the variation in composition at the species, subspecies, sex and individual level (Sweeney et al. 2004; Haribal et al. 2005; Mardon et al. 2010; Mihailova et al. 2014), it is reasonable to hypothesize that several proximate and ultimate mechanisms could be at play in influencing the composition of preen waxes in birds.

One interesting aspect of the function of preen waxes relates to the possible role that

olfactory-searching predators could play in shaping avian chemical profiles. It has been shown that some species of sandpipers (Family Scolopacidae) switch to a less volatile, diester rich preen wax profile during the breeding season (Reneerkens et al. 2002; Reneerkens et al. 2006), and this 'cryptic' wax is harder to detect for a mammal (Reneerkens et al. 2005). It has been hypothesized (Reneerkens et al. 2007) that incubating birds could switch to a less volatile preen wax to prevent a predator locating their nest via odorous cues, thus providing an extra layer of protection to a very sought-after prey (i.e., the eggs in the nest) in the form of “olfactory camouflage”. Following these publications, the switch from monoester-dominated to diester-dominated profiles has been shown in a few other species of birds (Thomas et al. 2010; Shaw et al. 2011; Tuttle et al. 2014). It is worth noting that a season- and sex- dependent pattern in preen wax composition had also been documented in mallard ducks *Anas platyrhynchos* (Jacob et al. 1979), which is consistent with the 'olfactory crypsis' hypothesis.

In this thesis, I examine a number of aspects of preen wax production and function in birds. I use a variety of approaches, including comparative analyses to examine broad scale patterns of preen wax composition, a study of the ontogeny of preen wax production in nestling birds, an experiment designed to test the role of diet on preen wax composition, and a study of whether aspects of an individual’s genetic make-up can be reflected in the preen wax odour profile. My overall objective is to add to the growing body of literature on the function and evolution of chemical communication in birds.

To date, the majority of studies on sociochemical signals in birds have focused on adults, and the authors investigating the olfactory crypsis hypothesis have also restricted their sampling to adult individuals. It is possible, though, that the evolutionary pressure for inconspicuousness of the odour profile may influence the ontogeny of preen wax production as well, resulting in chicks of species that have co-evolved with olfactory-searching predators to delay the onset of preen wax production and/or produce less volatile compounds while nestlings are at their most vulnerable. Conversely, where this pressure was absent, olfactory cues emitted by preen waxes could have been exploited for parent-offspring social communication (Cohen 1981; Célérier et al. 2011; Webster et al. 2015). I examined this hypothesis by sampling preen wax from several species of nestlings of both native New Zealand and introduced European species, from hatching to fledging. The resulting comparison of preen wax ontogeny is presented in **Chapter 2**.

One confounding factor that could influence a comparison of profiles in birds sampled in different habitats or at different times of the year is diet. It is evident that different species of birds sampled in New Zealand and in other locations could have access to different food sources (plants, insects, etc.), and the same is true for birds sampled in spring *vs.* winter, as most species will switch

diet in different seasons. If chemical profiles of preen wax are easily modified by changes in diet, it might be hard to detect the evolutionary signal due to predatory pressure for example. Moreover, the communication and recognition role of olfactory signatures – many examples of which are starting to emerge in the literature (see Célérier et al. 2011; Bonadonna & Sanz-Aguilar 2012; Leclaire et al. 2012; Bonadonna & Mardon 2013; Leclaire et al. 2014) - could be obscured if they are readily modified by external factors, such as changes in diet. As previously mentioned, the composition of preen wax is known to change seasonally in some species (Reneerkens et al. 2002), and it is important to tease out the possible role that season-associated dietary changes play in this shift. Despite the growing realisation that odours play an important role in social communication in birds, the robustness of olfactory signals to environmental factors, such as diet, has only been tested in a few studies. As a result, I set up a diet supplementation experiment in the wild, using silvereyes *Zosterops lateralis* as a model system. This species has a generalist diet, consuming a variety of foods such as fruit, insects and nectar, making it an interesting model to test the effect of diet on a wild passerine chemical profile. The results of this experiment are presented in **Chapter 3**.

It has so far been assumed that the socio-olfactory signals are endogenous in nature, and are related to an individual's genetic make-up, not only in birds but also in vertebrates in general (Willse et al. 2006; Bonadonna et al. 2007; Célérier et al. 2011; Leclaire & Merkling 2011). For example, it is known that odour cues can affect the preference for outbred males in females of wild mice (Ilmonen et al. 2009), and that an individual's scent can accurately signal the genetic quality (i.e. heterozygosity) in male lemurs (Charpentier et al. 2008; Charpentier et al. 2010;). However, the presence of a "heterozygosity signal" within the chemical signature has not been tested in birds so far. A reciprocal translocation of two populations of South island Robins (*Petroica australis*), carried out by my research group in the past to test the effectiveness of 'genetic rescue' using inbred donors (Heber et al. 2013), offered an ideal scenario to investigate the links between heterozygosity and avian preen wax profiles. I collected blood and preen wax samples from male South island robins on Motuara Island (New Zealand), where "inbred" and "outbred" birds coexist, and compared genetic diversity and chemical diversity across individuals: this study is presented in **Chapter 4**.

Finally, I test one of the predictions of the olfactory crypsis hypothesis using avian species assemblages from the South Pacific region. In contrast to continental species, passerine birds on many oceanic islands were not subject to selective pressure from mammalian predators that primarily use olfaction to locate prey, with the majority of predators being other birds, such as hawks or owl, that hunt primarily by sight. This was the case for New Zealand (Holdaway 1989) and New Caledonia (and other isolated islands), until exotic mammalian predators were introduced

by humans (Blackburn 2004; Diamond & Veitch 2011). If the switch in preen wax composition previously reported by Reneerkens et al. (2006) is indeed an evolutionary response to predatory pressure, I expect the majority of species sampled in New Zealand and New Caledonia to produce preen waxes that are more conspicuous than continental species, especially if, as it has been hypothesized, producing higher molecular weight, but less conspicuous preen waxes, incurs a cost for the individual (Reneerkens et al. 2006). Moreover, by comparing "island" species with their close "continental" (i.e. Australian) relatives, I can control for any potential phylogenetic effects in preen wax composition. Preliminary research from my group (Fluen 2008), examined the composition of preen waxes during the breeding and non-breeding season in four species of passerines native to New Zealand (bellbird *Anthornis melanura*, fantail *Rhipidura fuliginosa*, South Island robin *Petroica a. australis,* South Island saddleback *Philesturnus c. carunculatus*) and eleven introduced species (blackbird *Turdus merula,* chaffinch *Fringilla coelebs,* dunnock *Prunella modularis,* goldfinch *Carduelis carduelis,* greenfinch *C. chloris,* redpoll *C. flammea,* house sparrow *Passer domesticus,* yellowhammer *Emberiza citrinella* and silvereye *Zosterops lateralis*). The native species did not exhibit a switch to less volatile profiles, which was detected in the introduced species. It is important to note that the birds (native and introduced) were collected at the same site and during the same time periods, but because of the great phylogenetic distance among the two bird assemblages, it was not possible to conclusively ascribe the difference in volatility patterns to the different evolutionary histories of the native and introduced species. Thus, here I set up a comparative study including several closely related "island *vs.* continental" species pairs, in order to investigate the evolutionary role of predatory pressure on the shaping of preen wax composition. The results of this comparative study, involving 11 closely related species pairs ("island" and "continental" birds belong to the same genus or family) are presented in **Chapter 5**. Note that this project was undertaken in close collaboration with Aude Thierry: both of us have contributed to all aspects of the work, including sample collection at several locations, gas-chromatographic analysis, post-processing and statistical analysis of the data.

I conclude with a general discussion that brings together the findings from my research and I suggest approaches and avenues of development for future studies in the field of avian chemical ecology.

References

Amo, L. et al., 2008. Predator odour recognition and avoidance in a songbird. *Functional Ecology*, 22(2), pp.289–293.

Bang, B.G., 1960. Anatomical Evidence for Olfactory Function in Some Species of birds. *Nature*, 188, pp.547–549.

Bang, B.G., 1971. Functional anatomy of the olfactory system in 23 orders of birds. *Acta anatomica*, 79, p.1.

Bang, B.G. et al., 1968. The size of the olfactory bulb in 180 species of birds. *Auk*, 85, pp.55–61.

Blackburn, T.M., 2004. Avian Extinction and Mammalian Introductions on Oceanic Islands. *Science*, 305, pp.1955–1957.

Bonadonna, F. et al., 2007. Individual Odor Recognition in Birds: An Endogenous Olfactory Signature on Petrels' Feathers? *Journal of Chemical Ecology*, 33(9), pp.1819–1829. Available at: http://link.springer.com/10.1007/s10886-007-9345-7 [Accessed March 5, 2015].

Bonadonna, F. et al., 2004. Recognition of burrow's olfactory signature in blue petrels, *Halobaena caerulea*: An efficient discrimination mechanism in the dark. *Animal Behaviour*, 67(5), pp.893–898.

Bonadonna, F. & Bretagnolle, V., 2002. Smelling home: a good solution for burrow-finding in nocturnal petrels? *The Journal of experimental biology*, 205, pp.2519–2523. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12124375.

Bonadonna, F. & Mardon, J., 2013. Besides Colours and Songs, Odour is the New Black of Avian Communication. In M. L. East & M. Dehnhard, eds. *Chemical Signals in Vertebrates 12*. Springer New York, pp. 325–339. Available at: http://dx.doi.org/10.1007/978-1-4614-5927-9_26.

Bonadonna, F. & Sanz-Aguilar, A., 2012. Kin recognition and inbreeding avoidance in wild birds: the first evidence for individual kin-related odour recognition. *Animal Behaviour*, 84(3), pp.509–513. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0003347212002758 [Accessed January 21, 2014].

Burger, B.V. et al., 2004. Avian exocrine secretions. I. Chemical characterization of the volatile fraction of the uropygial secretion of the green woodhoopoe, *Phoeniculus purpureus*. *Journal of chemical ecology*, 30(8), pp.1603–1611.

Campagna, S. et al., 2012. Potential semiochemical molecules from birds: a practical and comprehensive compilation of the last 20 years studies. *Chemical senses*, 37(1), pp.3–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21798850 [Accessed September 28, 2014].

Caro, S.P. & Balthazart, J., 2010. Pheromones in birds: Myth or reality? *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, 196, pp.751–766.

Caro, S.P., Balthazart, J. & Bonadonna, F., 2015. The perfume of reproduction in birds: chemosignaling in avian social life. *Hormones and behavior*, 68, pp.25–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24928570 [Accessed September 3, 2014].

Caro, S.P., Balthazart, J. & Bonadonna, F., 2014. The perfume of reproduction in birds: Chemosignaling in avian social life. *Hormones and behavior*, 68, pp.25–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24928570.

Castro, I. et al., 2010. Olfaction in birds: a closer look at the kiwi (Apterygidae). *Journal of Avian Biology*, 41(3), pp.213–218. Available at: http://doi.wiley.com/10.1111/j.1600-

048X.2010.05010.x [Accessed January 24, 2014].

Célérier, A. et al., 2011. Chemical kin label in seabirds. *Biology letters*, 7(6), pp.807–10. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3210654&tool=pmcentrez&rendertype=abstract [Accessed March 5, 2015].

Charpentier, M.J.E. et al., 2010. Message "scent": lemurs detect the genetic relatedness and quality of conspecifics via olfactory cues. *Animal Behaviour*, 80(1), pp.101–108. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0003347210001351 [Accessed January 21, 2014].

Charpentier, M.J.E., Boulet, M. & Drea, C.M., 2008. Smelling right: the scent of male lemurs advertises genetic quality and relatedness. *Molecular ecology*, 17(14), pp.3225–3233. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18565115 [Accessed January 21, 2014].

Cohen, J., 1981. Olfaction and parental behavior in ring dove. *Biochemical Systematics and Ecology*, 9(4), pp.351–354.

Cunningham, S.J., Castro, I. & Potter, M.A., 2009. The relative importance of olfaction and remote touch in prey detection by North Island brown kiwis. *Animal Behaviour*, 78(4), pp.899–905. Available at: http://dx.doi.org/10.1016/j.anbehav.2009.07.015.

Diamond, J.M. & Veitch, C.R., 2011. Extinctions and Introductions in the New Zealand Avifauna: Cause and Effect ? *Science*, 211(4481), pp.499–501.

Fluen, T., 2008. *A Comparative Analysis Of Evolutionary Changes In Island Birds*. University of Canterbury, Christchurch, New Zealand.

Gagliardo, A., 2013. Forty years of olfactory navigation in birds. *The Journal of experimental biology*, 216(Pt 12), pp.2165–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23720797.

Graves, G.R., 1992. Greater yellow-headed vulture (*Cathartes melambrotus*) locates food by olfaction. *J Raptor Res*, 26, pp.38–39. Available at: www.scopus.com.

Grubb, T.C., 1973. Colony location by Leach's Petrel. *Auk*, 90(January), pp.78–82.

Gunderson, A.R., 2008. Feather-degrading Bacteria: A New Frontier in Avian and Host–Parasite Research? *The Auk*, 125(4), pp.972–979.

Gwinner, H. & Berger, S., 2008. Starling males select green nest material by olfaction using experience-independent and experience-dependent cues. *Animal Behaviour*, 75, pp.971–976.

Hagelin, J.C. & Jones, I.L., 2007. Bird odors and other chemical substances: a defense mechanism or overlooked mode of intraspecific communication? *The Auk*, 124(3), pp.741–761.

Haribal, M. et al., 2005. Chemistry of preen gland secretions of passerines: different pathways to same goal? why? *Chemoecology*, 15(4), pp.251–260. Available at: http://link.springer.com/10.1007/s00049-005-0318-4 [Accessed January 21, 2014].

Heber, S. et al., 2013. The genetic rescue of two bottlenecked South Island robin populations using translocations of inbred donors. *Proceedings of the Royal Society B: Biological Sciences*, 280(1752). Available at: http://www.ncbi.nlm.nih.gov/pubmed/23235701.

Holdaway, R.N., 1989. New Zealand's pre-human avifauna and its vulnerability. *New Zealand Journal of Ecology*, 12S, pp.11–25.

Ilmonen, P. et al., 2009. Females prefer the scent of outbred males: good-genes-as-heterozygosity? *BMC evolutionary biology*, 9, p.104. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2698884&tool=pmcentrez&rendertype=abstract [Accessed January 21, 2014].

Jacob, J., Balthazart, J. & Schoffeniels, E., 1979. Sex Differences in the Chemical Composition of Uropygial Gland Waxes in Domestic Ducks. *Biochemical Systematcs and Ecology*, 7, pp.149–153.

Leclaire, S. et al., 2014. Preen secretions encode information on MHC similarity in certain sex-dyads in a monogamous seabird. *Scientific Reports*, 4, p.6920.

Leclaire, S. et al., 2012. Semiochemical compounds of preen secretion reflect genetic make-up in a seabird species. *Proceedings of the Royal Society B: Biological Sciences*, 279(1731), pp.1185–93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3267147&tool=pmcentrez&rendertype=abstract [Accessed February 18, 2015].

Leclaire, S. & Merkling, T., 2011. An individual and a sex odor signature in kittiwakes? Study of the semiochemical composition of preen secretion and preen down feathers. *Naturwissenschaften*, 98, pp.615–624.

Leclaire, S., Wagner, R.H. & Hatch, S.A., 2009. Short communication. Can Kittiwakes smell? Experimental evidence in a Larid species. *Ibis*, 151, pp.584–587.

Leon, M. & Behse, J.H., 1977. Dissolution of the pheromonal bond: waning of approach response by weanling rats. *Physiology and Behavior*, 18, pp.393–397.

Mardon, J. et al., 2010. Species, gender, and identity: cracking petrels' sociochemical code. *Chemical senses*, 35(4), pp.309–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20190009 [Accessed January 21, 2014].

Martín-Platero, A.M. et al., 2006. Characterization of antimicrobial substances produced by Enterococcus faecalis MRR 10-3, isolated from the uropygial gland of the hoopoe (Upupa epops). *Applied and Environmental Microbiology*, 72(6), pp.4245–4249.

Mihailova, M. et al., 2014. Odour-based discrimination of subspecies, species and sexes in an avian species complex, the crimson rosella. *Animal Behaviour*, 95, pp.155–164. Available at: http://www.sciencedirect.com/science/article/pii/S0003347214002887.

Moyer, B.R., Rock, A.N. & Clayton, D.H., 2003. Experimental test of the importance of preen oil in rock doves (*Columba livia*). *The Auk*, 120(2), pp.490–496.

Muller-Schwarze, D., 2006. *Chemical ecology of vertebrates*, Cambridge University Press.

Nevitt, G. a & Bonadonna, F., 2005. Sensitivity to dimethyl sulphide suggests a mechanism for olfactory navigation by seabirds. *Biology letters*, 1(3), pp.303–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1617144&tool=pmcentrez&rendertype=abstract [Accessed September 24, 2014].

Petit, C. et al., 2002. Blue tits use selected plants and olfaction to maintain an aromatic environment for nestlings. *Ecology Letters*, 5(4), pp.585–589.

Rajchard, J., 2007. Intraspecific and interspecific chemosignals in birds: a review. *Veterinarni Medicina*, 2007(9), pp.385–391. Available at: http://agriculturejournals.cz/publicFiles/00350.pdf.

Reneerkens, J., 2007. *Functional aspects of seasonal variation in preen wax composition of sandpipers (Scolopacidae)*. Rijksuniversiteit Groningen, Netherlands

Reneerkens, J. et al., 2007. Parental role division predicts avian preen wax cycles. *Ibis*, 149(4), pp.721–729.

Reneerkens, J. et al., 2008. Seasonally changing preen wax composition: Red Knots' flexible defense against feather-degrading bacteria? *The Auk*, 125(2), pp.285–290.

Reneerkens, J., Piersma, T. & Damsté, J.S.S., 2006. Discerning adaptive value of seasonal variation in preen waxes: comparative and experimental approaches. *Acta Zoologica Sinica*, 52, pp.272–275.

Reneerkens, J., Piersma, T. & Damsté, J.S.S., 2002. Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why? *Proceedings of the Royal Society B: Biological Sciences*, 269(May), pp.2135–2139.

Reneerkens, J., Piersma, T. & Damsté, J.S.S., 2005. Switch to diester preen waxes may reduce avian nest predation by mammalian predators using olfactory cues. *The Journal of experimental biology*, 208, pp.4199–4202.

Roth, T.C., Cox, J. & Lima, S.L., 2008. Can foraging birds assess predation risk by scent ? *Animal Behaviour*, 76, pp.2021–2027.

Shaw, C.L. et al., 2011. Volatile and Semivolatile Compounds in Gray Catbird Uropygial Secretions Vary with Age and Between Breeding and Wintering Grounds. *Journal of Chemical Ecology*, 37, pp.329–339.

Shawkey, M.D., Pillai, S.R. & Hill, G.E., 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. *Journal of Avian Biology*, 34(4), pp.345–349.

Shumake, S.A., Smith, J.C. & Tucker, D., 1969. Olfactory intensity-difference thresholds in the pigeon. *Journal of comparative and physiological psychology*, 67(1), p.64.

Sieck, M.H. & Wenzel, B.M., 1969. Electrical activity of the olfactory bulb of the pigeon. *Electroencephalography and clinical neurophysiology*, 26(1), pp.62–69.

Soudek, S., 1927. The sense of smell in birds. In *X Congr. Internat. de Zool., Budapest*.

Stager, K.E., 1964. *The role of olfaction in food location by the turkey vulture (Cathartes aura)*, Los Angeles County Museum Contrib. Sci.

Stattelman, A.J., Talbot, R.B. & Coulter, D.B., 1975. Olfactory thresholds of pigeons (*Columba livia*), quail (*Colinus virginianus*) and chickens (*Gallus gallus*). *Comparative Biochemistry and Physiology Part A: Physiology*, 50(4), pp.807–809.

Sweeney, R.J., Lovette, I.J. & Harvey, E.L., 2004. Evolutionary variation in feather waxes of passerine birds. *Auk*, 121(2), pp.435–445. Available at: http://search.epnet.com/login.aspx?direct=true&db=aph&an=13377085.

Thomas, R.H. et al., 2010. Use of TLC-FID and GC-MS／FID to examine the effects of migratory state, diet and captivity on preen wax composition in White-throated Sparrows *Zonotrichia albicollis*. *Ibis*, 152, pp.782–792.

Tucker, D., 1965. Electrophysiological evidence for olfactory function in birds.

Tuttle, E.M. et al., 2014. Variation in preen oil composition pertaining to season, sex, and genotype in the polymorphic white-throated sparrow. *Journal of Chemical Ecology*, pp.1025–1038.

Walter, W.G., 1943. Some experiments on the sense of smell in birds. *Arch. néerl. Physiol.*, 27, p.172.

Webster, B., Hayes, W. & Pike, T.W., 2015. Avian Egg Odour Encodes Information on Embryo Sex, Fertility and Development. PloS one, pp.1–10.

Wenzel, B.M., 2007. Avian olfaction: Then and now. *Journal of Ornithology*, 148(S2), pp.191–194. Available at: http://link.springer.com/10.1007/s10336-007-0147-z [Accessed March 19, 2015].

Wenzel, B.M., 1971. Olfactory sensation in the kiwi and other birds. *Annals of the New York Academy of Sciences*, pp.183–193.

Wenzel, B.M. & Sieck, M.H., 1972. Olfactory Perception and Bulbar Electrical Activity in Several Avian Species. *Physiology and Behavior*, 9, pp.287–293.

Whittaker, D.J. et al., 2009. Behavioral responses of nesting female dark-eyed juncos *Junco hyemalis* to hetero- and conspecific passerine preen oils. *Journal of Avian Biology*, 40, pp.579–583.

Willse, A. et al., 2006. Individual odortypes: interaction of MHC and background genes. *Immunogenetics*, 58(12), pp.967–982. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17089117 [Accessed January 21, 2014].

Wyatt, T.D., 2003. *PHEROMONES AND ANIMAL BEHAVIOUR*, Cambridge University Press.

Zhang, J.-X. et al., 2010. Uropygial gland-secreted alkanols contribute to olfactory sex signals in budgerigars. *Chemical senses*, 35(5), pp.375–382. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20212012 [Accessed January 21, 2014].

Zhang, Y.-H., Du, Y.-F. & Zhang, J.-X., 2013. Uropygial gland volatiles facilitate species recognition between two sympatric sibling bird species. *Behavioral Ecology*, 24(6), pp.1271–1278.

Chapter 2

Preen wax production ontogeny in nestlings of introduced and native New Zealand species.

Abstract: *I explored the ontogeny and variability of preen wax profiles in nestlings of introduced and native New Zealand passerine species, by comparing the onset of preen wax production and analyzing the chemical profiles of preen wax samples repeatedly during the nestling stage. The onset of preen wax production did not vary between native and introduced species. I extracted three summary variables from the chemical profiles obtained by gas chromatographic analysis of preen wax: minimum retention time, maximum retention time, and number of peaks above the instrument detection limit, which were all significantly correlated with each other. I tested the effect of status * nestling age on minimum retention time, and found that there was a significant increase in minimum retention time as nestlings of introduced species grew older, while minimum retention time tended to decrease with increasing age in native nestlings. Multivariate analysis of the profiles revealed significant variation at all the levels examined (status, species and nest). The introduced European starling in particular showed a very distinctive profile, with an abundance of volatiles in the early nestling phase, which may be linked to an anti-parasitic function. The preliminary nature of this study does not allow me to reach definitive conclusions, but I hypothesize that preen wax in introduced nestling may have evolved to respond to the necessities of protection against parasites in the early nestling stages, and inconspicuousness to predators in the late stages. The latter did not appear to have an influence on the evolution of preen waxes in native species, whose more volatile profile manifests itself at the late nestling stages already.*

Introduction

Chemical communication in birds has been the focus of much scientific research, with a surge of publications on this topic in recent years (see Figure 2.1). Studies have focused on sex differences, individual recognition (Bonadonna et al. 2007), kin recognition (Bonadonna & Sanz-Aguilar 2012), species recognition (Zhang et al. 2013), environmental influences (Haribal et al. 2009), phylogenetic variation (Sweeney et al. 2004, Soini et al. 2013) and have covered many aspects of both avian behaviour and ecology. Of these studies, though, only very few involve nestlings. In fact, a search query on Web of Science database (version 5.16.1) for the keywords 'preen wax AND nestling*'[1] only returned 3 results. Moreover, two of these articles focus on hoopoe (*Upupa epops*) nestlings, which are very peculiar in that their uropygial secretions are brown and malodorous, like those of incubating females but unlike non-incubating conspecific or heterospecific adults (Soler et al. 2008; Martín-Vivaldi et al. 2009). This unusual composition seems to be related to the presence of specific strains of symbiotic bacteria in the uropygial gland (Martín-Vivaldi et al. 2010), which are not found in other species which produce the more typical white and less odorous preen wax (Soler et al. 2008), making it hard to generalize from this example.

Nestlings possess a uropygial gland (the gland responsible for the production of preen wax) that is visible from birth, but nothing is known about the onset of preen wax production, or the composition of preen wax produced by nestlings versus juvenile and adult birds. In domestic pigeons (*Columba livia*), the inner epithelial lining of the uropygial gland develops soon after hatching but atrophies as the chick stops receiving pigeon milk (Esther, 1930 cited in Elder, 1954). In Eider ducks (*Somateria mollissima*), the gland only becomes functional several days after hatching (Madsen, 1941 in Elder, 1954) and Amo et al. (2014) suggested that in Spotless starlings (*Sturnus unicolor*) the gland is closed and inactive around 5-6 days of age, but becomes open and active at 12 days of age. Nevertheless, little is known about the development of preen wax production in nestling birds and there are no systematic studies on the ontogeny of preen wax production nor how this varies across species.

[1] Web of Science™ "Advanced Search" performed on 10/04/2015. Search query: TS=('preen wax AND nestling*'). Number of hits: 3. I tried to increase the number of hits by broadening the search with query: TS=('preen wax AND nestling*' OR 'chemical communication AND nestling*'). This returned 9 hits, but the only relevant publications were the 3 returned by the original search. Relevant publications are defined as publications that address preen wax production or chemical communication in avian nestlings.

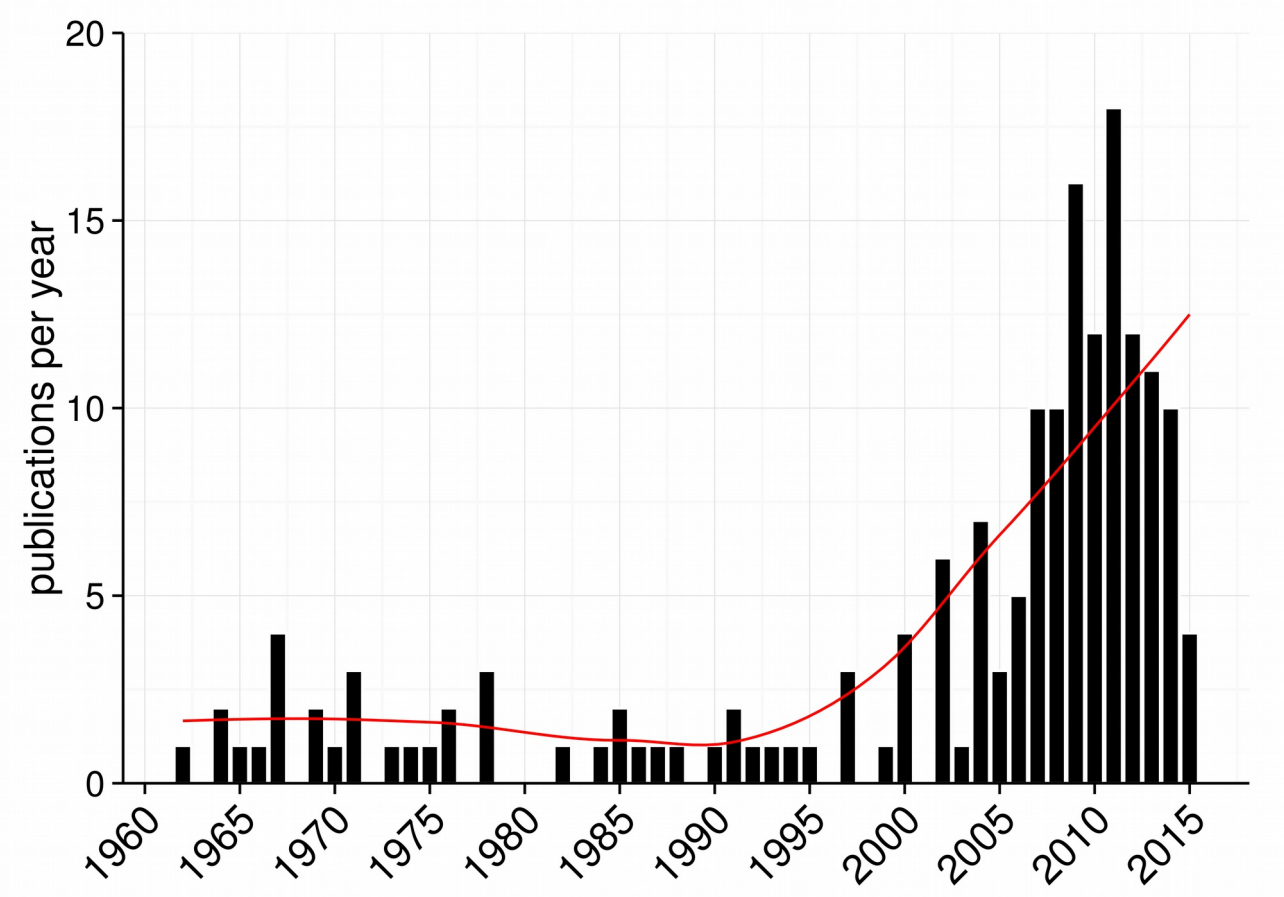

Figure 2.1: Temporal development of the published scientific literature in the field of avian chemical communication. Histogram of the results of the search query TS=('preen wax' OR 'chemical communication AND bird*') carried out in Web of ScienceTM on 10/04/2015.

A better understanding of nestling preen wax production (and any resulting odours produced by the preen wax) would make an important contribution to some of the topics explored in avian chemical ecology in the past decade. For instance, many studies on procellariiform seabirds have reported the occurrence of re-homing based on nest odour cues (Bonadonna et al. 2001; Bonadonna & Bretagnolle 2002; Bonadonna et al. 2004), but it is generally understood that the adults are the source of the nest smell, either via uropygial secretions or other sources (i.e. feathers, faeces, other bodily excretions), particularly as it appears that at least one species where nestlings show olfactory-based natal nest recognition (British storm-petrel, *Hydrobates pelagicus*) does not possess parent-offspring recognition (Minguez 1997), making the nestlings' odour unlikely to be an efficient signal for re-homing. On the other hand, chicks of this species choose their own body odour over that of a conspecific, pointing to the possibility of the individual odour profile of a bird being developed quite early in life (de Leon et al. 2003). Adults of other species, such as Antarctic prion *Pachyptyla desolata*, are able to recognize their own nest during the egg incubation phase (Bonadonna et al. 2003), and most experiments on natal nest recognition by adults have been

carried out at this stage, which is possibly why the contribution of nestlings to the nest odour has been underestimated.

Olfactory-based natal nest recognition has been detected in diurnal passerine species as well (Caspers & Krause 2011; Krause & Caspers 2012). Here, both adults and fledglings of estrildid finches have been tested, and it has been suggested that nestlings contribute to the nest odour that is being used as a cue (Krause & Caspers 2012). The existence of a "nestling odour" in this songbird family is also supported by a study on kin-recognition (Krause et al. 2012), although the experimental design includes the contribution of parents to the nest odour, and so the separate effects of nestlings and parents cannot be distinguished. However, the strength of the preference of nestlings for their nest was positively correlated with the number of genetically related individuals in the nest (i.e. the higher the number of full siblings, the stronger the preference), suggesting nestling odours alone can potentially act as cues for nest recognition in this species (Krause et al. 2012).

Nestling odour could also be a factor in nest detection by olfactory-searching predators, contributing to the risk of nest predation. Olfactory cues from the nest are known to contribute to nest detection and to facilitate nest predation by olfactory-searching predators (Whelan et al. 1994; Borgo 2008), but studies on the topic have so far only focused on odour sources such as feathers and faeces (Coppedge et al. 2007, Price and Banks 2012). It is reasonable to think that nestlings too would contribute to the nest odour, possibly increasing overall detectability of the nest to predators. Indeed, olfactory crypsis has been proposed as an explanation for the switch to less volatile esters in the preen wax of incubating adult sandpipers (Reneerkens et al. 2005), a species that is greatly affected by predation from olfactory-searching mammals (Blomqvist et al. 2002). Olfactory camouflage in the incubating and brooding parent(s) could lower nest detectability, but the benefits would only be partial if nestlings themselves contribute to the nest odour, unless their also modified the composition of their preen wax to reduce detectability. Moreover, for some species of altricial passerines, predation at the nestling stage can be much higher than at the egg stage, and seems to increase with nestling age (Pietz & Granfors 2000). As nest predation is the primary cause of nest failure in most birds (Ricklefs 1969; Martin 1993), it is important to investigate any possible contributing factor that increases this risk, including potential olfactory cues.

Finally, preen wax could play a role in nestling antimicrobial defense. Preen wax inhibits the growth of feather-degrading bacteria in the lab (Shawkey et al. 2003; Reneerkens et al. 2008), and it is also active against other types of potentially damaging organisms, such as ectoparasites (Moyer et al. 2003) and fungi (Bandyopadhyay & Bhattacharyya 1999). For some species, such as European hoopoe and woodhoopoe (*Phoeniculus purpureus*), specific volatile substances harbouring anti-

microbial activity have been identified (Martín-Platero et al. 2006; Rajchard 2010). As parasites lower reproductive success of a nest and negatively influence a nestling's body condition (Richner et al. 1993; Tschirren et al. 2003), we would expect antimicrobial and antiparasitic function of preen wax to manifest at the nestling stage as well.

In this chapter, I investigate the timing of production of preen wax in nestlings as well as changes in the composition of their preen wax in a variety of New Zealand passerine birds. My study includes both native New Zealand species as well as species introduced from Europe. The native and introduced birds in New Zealand that I studied in this chapter now share a common environment (native forest), but have very different evolutionary histories. Until introduced by humans, New Zealand lacked any predatory mammals, a guild of predators that make extensive use of olfactory cues for locating their prey. In contrast, native birds were subject to predation primarily by visually hunting predators (e.g. falcons). Thus, I hypothesized that, like adults (see chapter 5), nestlings from native New Zealand species and introduced exotic species have faced different selective pressures and constraints on their preen wax phenotype. The absence of olfactory-searching predators during the evolutionary history of New Zealand species may have shaped their preen wax composition and the onset of preen wax production in different ways compared to birds that were exposed to high rates of mammalian or reptilian predators (such as was the case for introduced European birds in their native range). If native birds are not only behaviorally naïve towards predators, but also "chemically naive", this might play an important role in their heightened susceptibility to invasive predators compared to introduced species, which have evolved in sympatry with those predators.

In order to investigate the composition of preen wax in nestlings, I followed the development of broods of several native and introduced species, and took repeated preen wax samples from chicks.

Materials and methods

Study site and species

I carried out this study in a regenerating native forest on the east coast of the South Island, New Zealand (Kowhai Bush Reserve, 173°36'E, 42°23'S), in October-December 2012. I located nests 5 species of passerine birds (2 native and 3 introduced; see list in Table 2.1) in the early incubation stage, and visited them every other day to determine hatching date. After hatching, I revisited nests every other day, taking measurements from the chicks and attempting to extract preen wax. I recorded the date when preen wax extraction was first successful, and thereafter collected samples by gently squeezing the uropygial gland with forceps with wax-coated tips. The preen wax extruded

was then collected on a clean stainless-steel inoculation loop and placed in a clean glass insert lodged inside a glass vial, sealed with teflon-covered lids to minimize evaporation or contamination of samples. Vials were kept cool using freezer packs in the field for a maximum of 8 hours, until later frozen. Samples were kept at -20°C until analysis. I collected samples from nestling until fledging, or until it appeared that manipulating nestlings would cause nestlings to prematurely fledge (based on enhanced nestling activity during nest observations prior to sampling and personal knowledge of proclivity to force fledging in certain species e.g. fantail).

Sample preparation and analysis

Samples were dissolved in 100 µl of ethyl acetate, poured directly into the insert containing the inoculation loop and the preen wax. The vial was then re-capped, and vortexed for 60 seconds at 700 rpm to ensure dissolution of the preen wax. The original cap was then substituted with a chromatographic cap fitted with a single-use PTFE silicone septum. Samples were analysed on a Shimadzu GC-2010 gas chromatograph, equipped with a Shimadzu AOC-20i+s auto-injector and a Varian CP-SIL 5 CB capillary column (25 m length x 320 µm internal diameter x 0.12 µm film thickness). Injection volume was 1 µl, with a 6:1 split ratio. Injection port temperature was set at 250°C, the carrier gas was nitrogen with a total flow of 19.0 ml/min and a linear velocity of 36.7 cm/sec. The FID detector operated at 320°C, with a sampling interval of 40 msec. Oven temperature was programmed as follows: initial temperature 70°C with a hold time of 4 mins, then increased to 130°C at a rate of 20°C/min, and finally increased to 320°C at 4°C/min rate (hold time 15 mins). Results were recorded on Shimadzu's GCSolution, version 2.3 (©Shimadzu 2002-2009) software.

Statistical methods

I recorded the onset of preen wax production (or excretion) as nestling age in days (referred below as “absolute start of preen wax production”), and also calculated the onset of preen wax production as proportion of nestling stage length. All data on the duration of nestling stage for the species examined was obtained from the Handbook of Australian, New Zealand and Antarctic Birds, Volumes 5 and 7.

I extracted three synthetic measures from each profile, so that I could compare the profiles of nestlings of different ages using univariate statistics: the measures are minimum retention time, maximum retention time, and number of peaks above detection limit of the instrument.

I then conducted a multivariate analysis of variance (PERMANOVA) on the preen wax profiles of nestlings of 5 species (sample size and species detailed in Table 2.1). The alignment of the peaks has to be taken with caution as, in order to perform this analysis, I had to align profiles across

species, and this was done based on retention time and profile similarity, rather than precise molecular information on the compounds themselves. Nevertheless, the retention times provide valuable information on the volatility of the peaks, which is valid across species. Peaks that were present on a chromatogram but fell below the detection limit of the instrument were given an arbitrary value of 0.000001. Only peaks that were above the detection limit for the instrument in at least 5% of the samples were included in the analysis. The area of each peak was converted to its proportional contribution to total peak area in that sample, and PERMANOVA was carried out using chord distances among samples.

Due to the nestedness of my experimental design (nests within species within status), I modified the function adonis native to the R package vegan, to allow the PERMANOVA pseudo-F ratios for status and species to be calculated using the appropriate error structure for each factor. The native function provides incorrect estimates of the pseudo-F ratios for all but the innermost nested factor (nest in this case), as it uses the mean squares for the global residuals as denominator for each F-ratio. The ad-hoc function I created uses the mean square for the nested factor to calculate the F-ratio for the overlying factor (in my case, MS nest in used as denominator for the

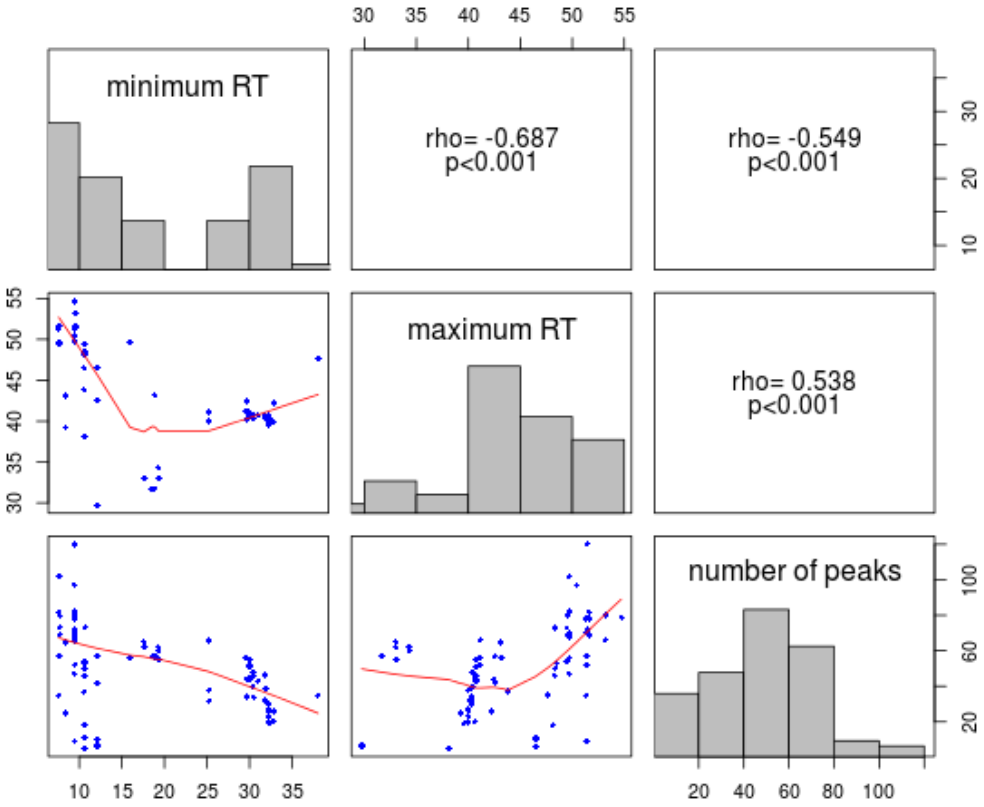

Figure 2.2: Correlation, distribution and scatterplot matrices for the three measures extracted from the preen wax profiles. On the upper half of the plot, rho and P values for Spearman's correlation between the 3 variables are visible. On the lower half, scatterplot of the reciprocal distribution of the values for each variable are shown; histograms for each single variable are placed on the diagonal.

calculation of *species* F-ratio, and MS species is used to calculate the *status* F-ratio). The native adonis function was used to calculate the F-ratio for nest. My main interest was analysing the effect of "status" on the preen wax profiles of the nestlings, and species and nests were my nested factors, not so central to my hypothesis: to better visualize the main effect (status), I set up a biplot (Figure 2.9) using CAP scores (CAP scores were obtained via *capscale* function in R, which is a constrained version of metric scaling, and is therefore useful to visualise the contribution of the main effect, i.e. status, on the ordination of chemical profiles).

Table 2.1: Species and sample sizes used in the multivariate analysis of variance of the nestlings' chemical profiles. Sample size is the number of nestlings analysed.

Status	Common name	Scientific name	Sample size
Introduced	Blackbird	*Turdus merula*	6
Introduced	Song thrush	*Turdus philomelos*	5
Introduced	Starling	*Sturnus vulgaris*	28
Native	NZ Fantail	*Rhipidura fuliginosa*	20
Native	Rifleman	*Acanthisitta chloris*	10

Results

The onset of preen wax production did not vary significantly by status (native versus introduced species), but it did show significant variation among species, both in terms of absolute start of preen wax production (nested ANOVA: status: $F_{1,3} = 0.001$, $P = 0.981$, species: $F_{3,39} = 36.16$, $P < 0.000001$) and in terms of proportion of the nestling stage at which production occurs (nested ANOVA: status: $F_{1,3} = 0.278$, $P = 0.635$, species: $F_{3,39} = 18.04$, $P < 0.000001$). Proportions were logit transformed to deal with departure from normality within some of the groups.

Table 2.2: Summary statistics for onset of preen wax production by species

Status	Common name	Start of preen wax production (days, mean ± s.d.)	Start of preen wax production as proportion of the nestling phase length
Introduced	Blackbird	8.6 ± 1.3	0.70
Introduced	Song thrush	11.8 ± 1.2	0.90
Introduced	Starling	8.6 ± 1.6	0.47
Native	NZ Fantail	8.2 ± 1.1	0.58
Native	Rifleman	13.5 ± 1.2	0.56

The three measures extracted from the profiles were significantly correlated with each other (see

Figure 2.2), so I tested only the minimum retention retention time against status * nestling age (using “Species” as the Error term for the ANOVA model). Model residuals were checked against fitted values for homoscedasticity and against theoretical quantiles for normality. All terms of the model were significant, including the status * nestling age interaction (ANOVA: status: $F_{1,1}$ = 11964, P = 0.006, nestling age: $F_{1,1}$ = 8802, P = 0.007, status * nestling age: $F_{1,1}$ = 1495, P = 0.01, see Figure 2.3). I therefore investigated the relationship between minimum retention time and nestling age for native and introduced birds separately, by fitting a mixed model via function lme (R package nlme), and using species as a random factor. The relationship between minimum retention time and nestling age was not significant for native birds ($F_{1,31}$ = 0.51, P = 0.47) but it was significant for introduced birds ($F_{1,34}$ = 4.74, P = 0.03). Example traces of native and introduced nestlings at different ages can be seen in Figures 2.4, 2.5, 2.6 and 2.7.

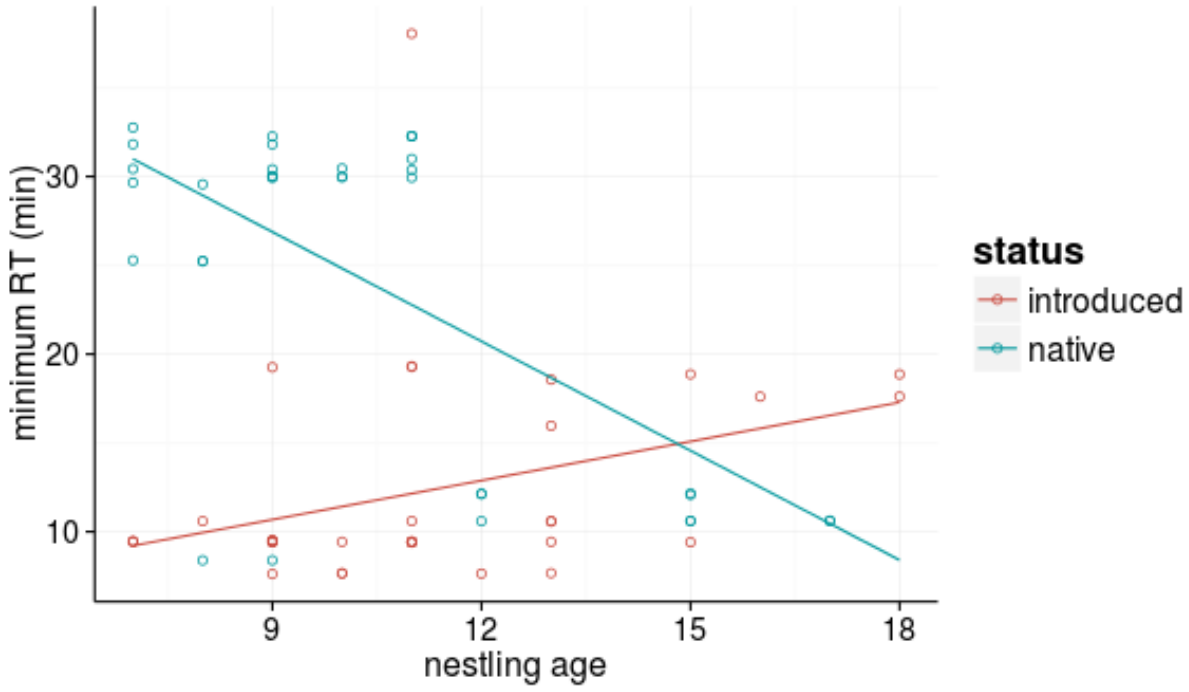

Figure 2.3: Scatterplot of minimum retention time (in minutes) extracted from each nestling's chromatographic trace, plotted against the nestling's age. The presence of a status * age interaction is clearly identifiable by observing the linear regression lines, fitted for the two levels of the factor status.

The multivariate analysis of the profiles revealed significant variation at all levels examined: this was more pronounced at the species and status level than at the nest level (adonis, status: pseudo-$F_{1,3}$ = 1.82, R^2 = 0.19, P < 0.0001, permutations = 9999; species: pseudo-$F_{4,13}$ = 10.32, R^2 = 0.61, P < 0.0001, permutations = 9999; nest: pseudo-$F_{13,51}$ = 1.29, R^2 = 0.14, P = 0.01, permutations = 9999; see also Figure 2.8 for the histograms of the distribution of pseudo-F values under permutation for the three factors and Figure 2.9 for a 2-dimensional plot of the scores of the constrained analysis on

status). Moreover, it is important to stress that the test for multivariate homogeneity of group dispersion was significant at the nest level (betadisper, nest: pseudo-$F_{17,51}$ = 2.6401, P= 0.004); as PERMANOVA and adonis are sensitive to both differences in variation and dispersion (Anderson 2001), the significant result might be driven both by a difference in multivariate location and in multivariate dispersion among nests. At the status and species level, there was no significant difference in multivariate group dispersion (betadisper, status: pseudo-$F_{1,67}$ = 0.0012, P= 0.9728; species: pseudo-$F_{4,64}$ = 0.3432, P= 0.8478).

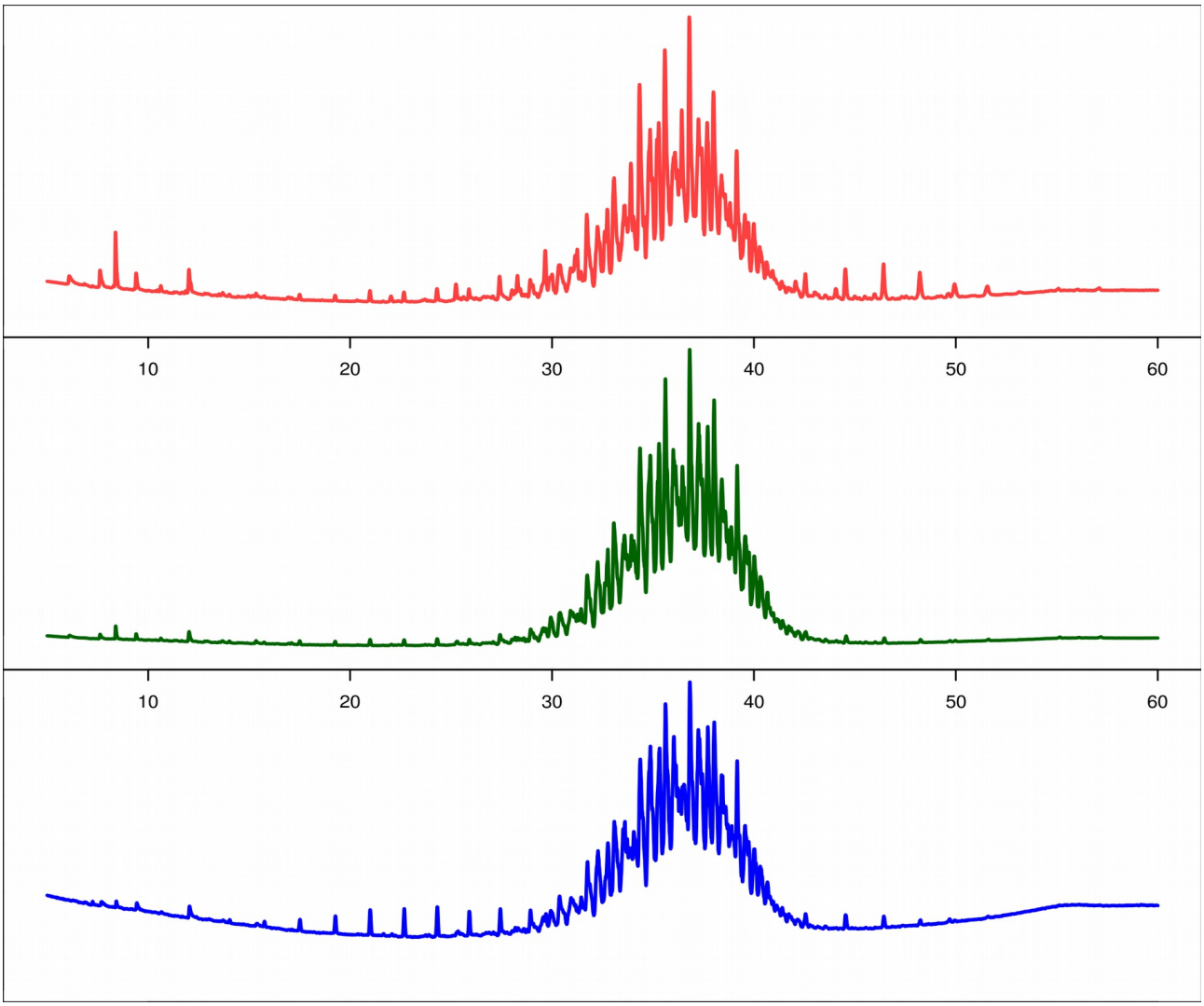

Figure 2.4: Examples of chromatographic traces (retention time in minutes on the x axis, trace intensity on the y axis) from NZ fantail nestlings, aged 7 days (red), 9 days (green) and 11 days (blue).

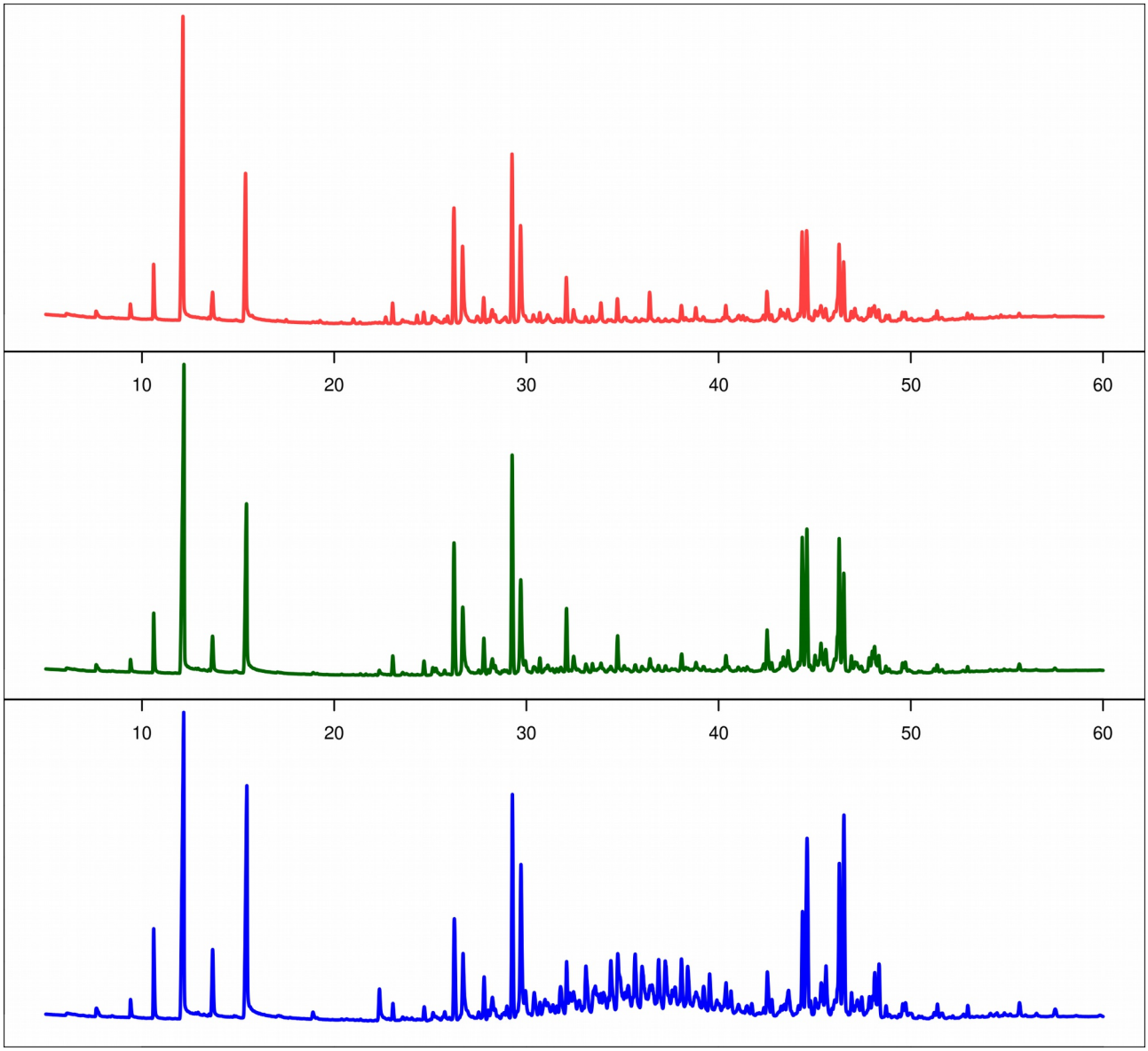

Figure 2.5: Examples of chromatographic traces (retention time in minutes on the x axis, trace intensity on the y axis) from Rifleman nestlings, aged 12 days (red), 15 days (green) and 17 days (blue).

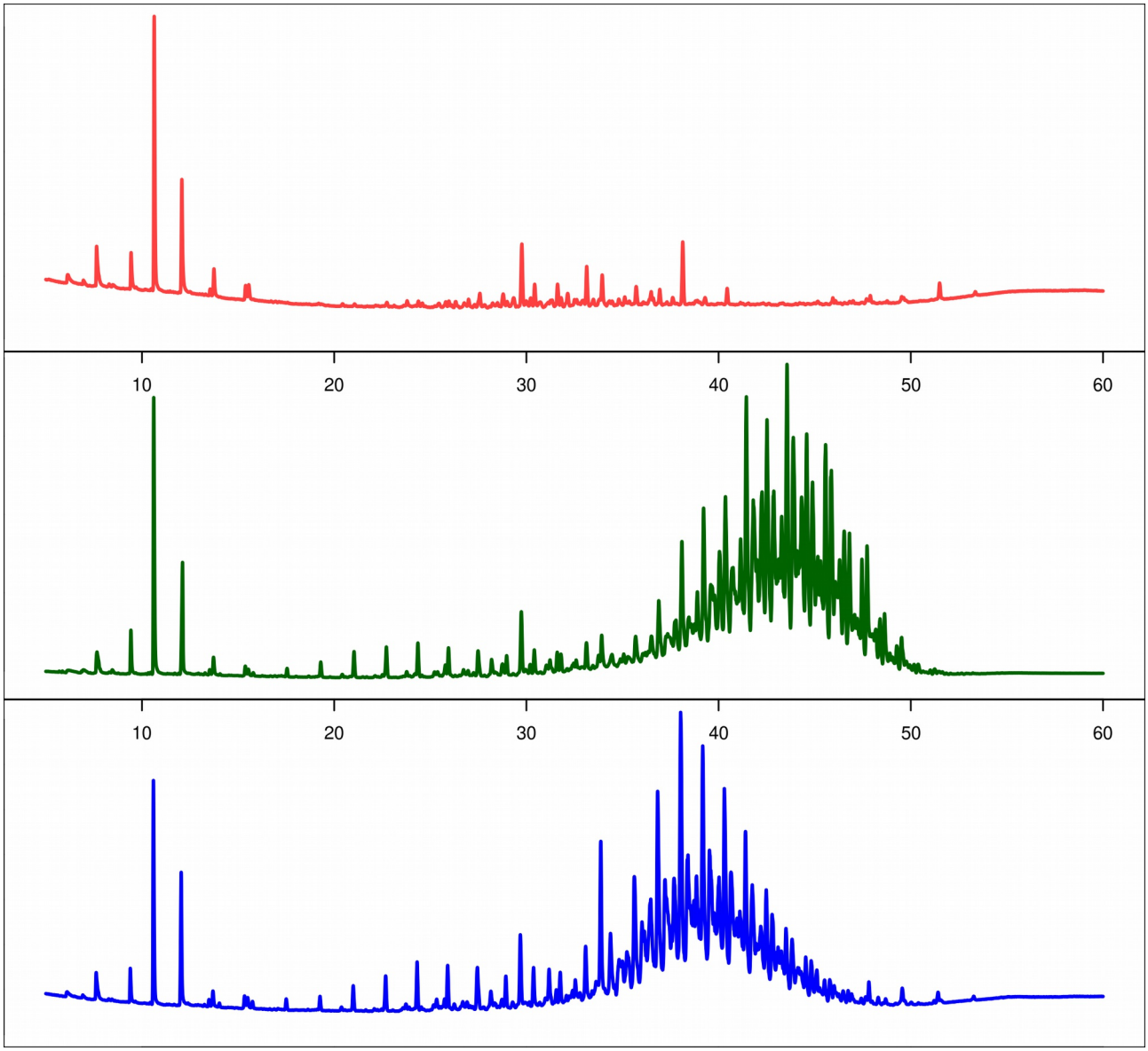

Figure 2.6: Examples of chromatographic traces (retention time in minutes on the x axis, trace intensity on the y axis) from Blackbird nestlings, aged 8 days (red), 10 days (green) and 13 days (blue).

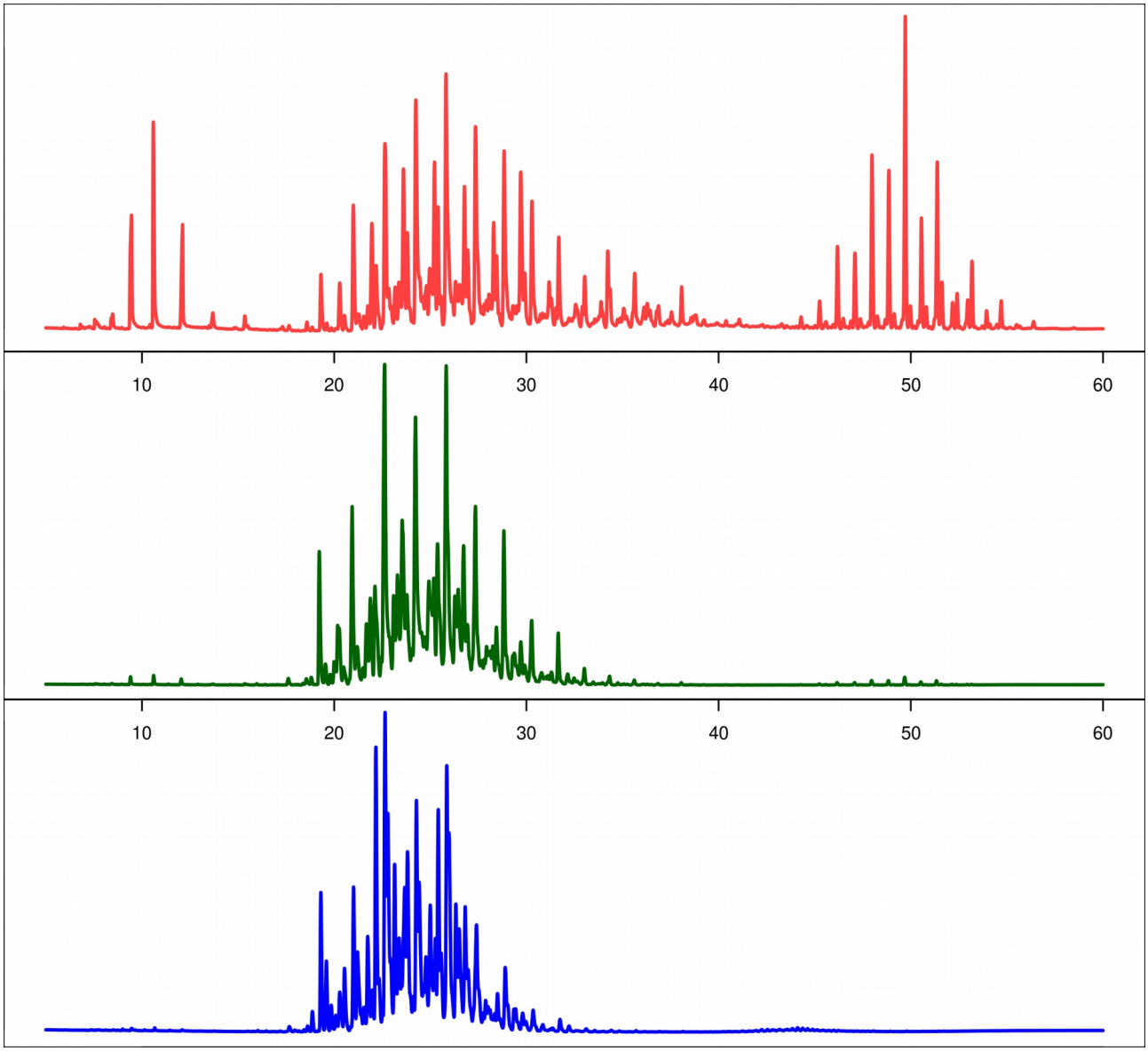

Figure 2.7: Examples of chromatographic traces (retention time in minutes on the x axis, trace intensity on the y axis) from Starling nestlings, aged 7 days (red), 11 days (green) and 18 days (blue).

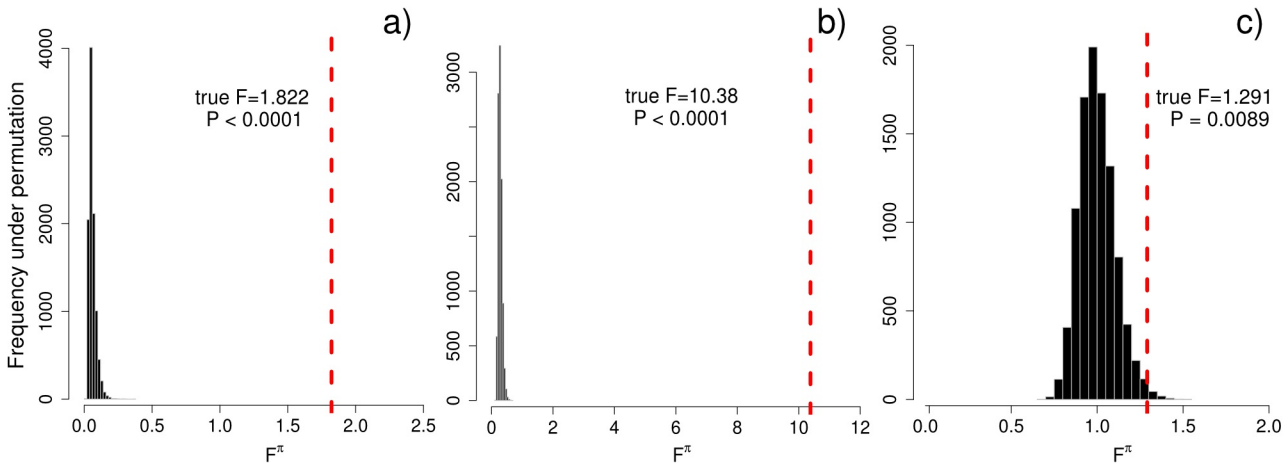

Figure 2.8: Distribution of PERMANOVA pseudo-F statistic (9999 permutations) for a) status b) species and c) nest.

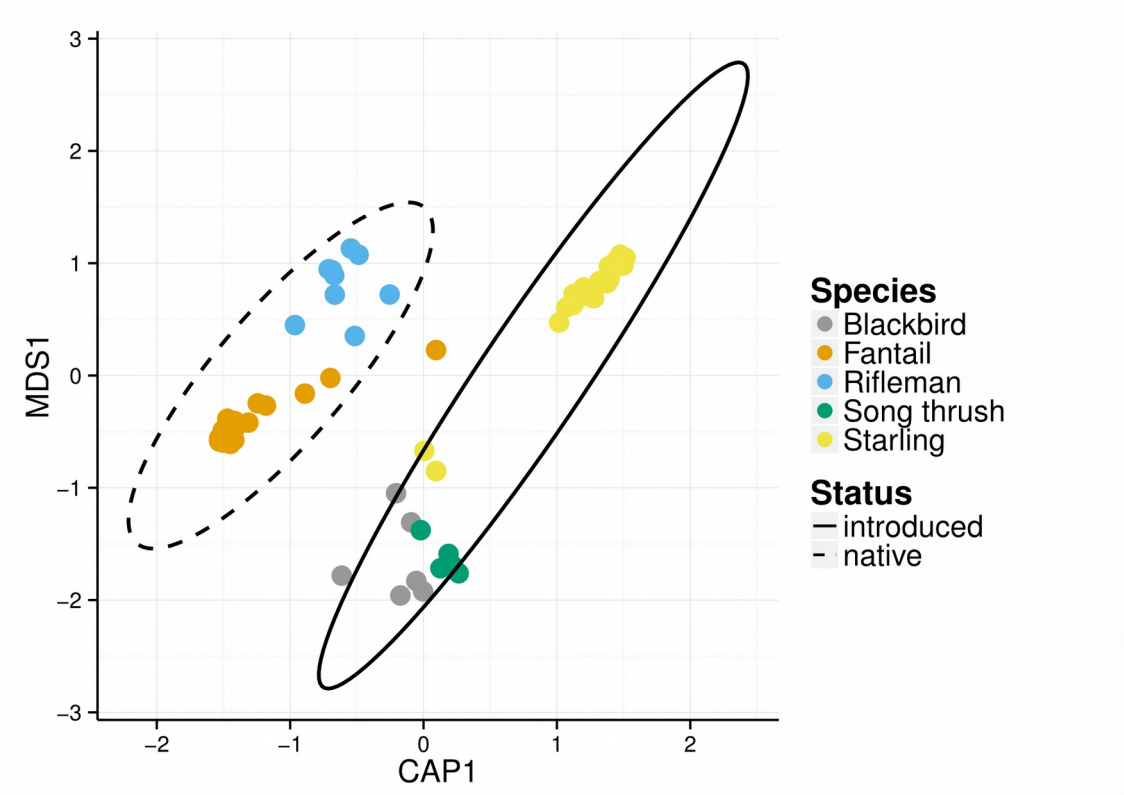

Figure 2.9: CAP plot from constrained analysis (factor: status) of nestlings' preen wax profiles.

Discussion

This study has shown that considerable variation exists among the preen wax profiles of some passerine species, already manifest at the nestling stage. I expected to find that native island species would commence preen wax production earlier in the nestling phase than introduced species, as the lack of predators during their evolutionary history might have relaxed selection on camouflaging

the nest odour and encouraged early preen wax production that could potentially have communication or anti-parasitic advantages for the nestlings (Martín-Vivaldi et al. 2010). However, this was not supported by my analysis, even though the two native species I examined (Rifleman and NZ Fantail) showed earlier proportional onset of preen wax production than 2 of the 3 introduced species. This is because one of the introduced species, the European starling, had the earliest proportional start of preen wax production, and also clustered farthest away from the other species in the unconstrained ordination plot, pointing to the distinctiveness of its profile.

As chemical signals are known to play a role in communication in the congeneric Spotless starling, *Sturnus unicolor* (Amo et al. 2012), selection on the use of chemical signals by nestlings might have played a role in favouring an early start of preen wax production. This may be especially important in species such as starlings which experience intense within-brood competition (Gil et al. 2008, HANZAB Vol. 7) and frequent intra-specific parasitism (Evans 1988; Lombardo et al. 1989). Another reason for early production of preen wax could be the prevalence of parasites in starling nests. Starlings have very high rates of infection by ectoparasites (Boyd 1951) and the individuals sampled showed higher macro-ectoparasite load and worse body condition (i.e. more visible scabs) than nestlings of the other species (personal observation). It has been hypothesized that the peculiar aromatic environment males create within the nest by decorating it with herbs could function as insect repellent, as has been shown in blue tits *Parus caeruleu*s, which seem to gain protection from blood-sucking insects by deploying aromatic herbs within their nests (Lafuma et al. 2001; Scott-Baumann & Morgan 2015). Studies on starlings, though, have so far failed to support the hypothesis that green plants have a nest protection activity (Fauth et al. 1991; Gwinner et al. 2000), with a role as sexual signal being currently favoured in the literature (Scott-Baumann & Morgan 2015).

The high prevalence of parasites in starlings nests does constitute a strong selective environment, though, and the anti-parasitic function, if not fulfilled by aromatic plants, could be carried out by chemical compounds present in preen wax. Detailed comparison of preen wax profiles across species has not been carried out yet for the nestling samples (as this requires confirming the identity of compounds via GC-MS), but based on retention times and profile shapes, starling profiles appear markedly different from the other species, and contain a higher number of early eluting compounds. It is also interesting to notice that, as shown in Figure 2.7, the youngest nestlings display a much richer profile than the older ones. Studies on other species have shown that, it is typically the volatile fraction of the preen wax that is thought to have anti-microbial, or antiparasitic activity (Jacob et al. 1997; Burtt 1999; Burger et al. 2004). The antiparasitic function might be crucial in the early stages of chick life, to be supported or superceded in the late stages by

anti-predatory adaptations: as previously mentioned, there are indications that predation rates increase with nestling age (Pietz and Granfors 2000), and at a later stage, i.e. towards fledging time, it might be more crucial for a nestling to be inconspicuous to predators rather than protected by parasites. This seems to be reflected in the significant increase in minimum retention times with age in the profiles of introduced birds, meaning that the odour signals progressively lose their most volatile fraction as the nestlings grow older. This is not true for native species, where I found a non-significant trend in the opposite direction: if anything, the profiles become more volatile as the birds progress through the nestling phase (Figure 2.3, 2.4). This is in agreement with Fluen's (2008) findings on adults, and also with what reported here in Chapter 5: the tendency to a higher volatility in the profiles of native species seem to manifest itself in the late nestling stages already, offering one more indication that these volatile profiles might be important for communication in the later stages of life, as indicated by experiments on sociochemical communication in a native New Zealand species (Thierry 2014). Unfortunately, an increase in the volatility of preen wax of native species may make nests (and nestlings) increasingly more conspicuous to predators that use olfactory cues to locate their prey. Such an increase in conspicuousness may have been advantageous in the New Zealand environment before the arrival of humans, but now may add to their vulnerability to the predators we have introduced.

References

Amo, L. et al., 2012. Sex recognition by odour and variation in the uropygial gland secretion in starlings. *The Journal of animal ecology*, 81(3), pp.4–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22220811 [Accessed January 21, 2014].

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, 26, pp.32–46.

Anderson, M.J and Santana-Garcon, J., 2015. Measures of precision for dissimilarity-based multivariate analysis of ecological communities. *Ecology Letters*, 18, pp.66-73.

Bandyopadhyay, A. & Bhattacharyya, S.P., 1999. Influence of fowl uropygial gland and its secretory lipid components on the growth of skin surface fungi of fowl. *Indian Journal of Experimental Biology*, 37(12), pp.1218–1222.

Blomqvist, S. et al., 2002. Indirect effects of lemming cycles on sandpiper dynamics: 50 Years of counts from southern Sweden. *Oecologia*, 133(2), pp.146–158.

Bonadonna, F. et al., 2007. Individual odor recognition in birds: An endogenous olfactory signature on petrels' feathers? *Journal of Chemical Ecology*, 33, pp.1819–1829.

Bonadonna, F. et al., 2004. Recognition of burrow's olfactory signature in blue petrels, *Halobaena caerulea*: an efficient discrimination mechanism in the dark. *Animal Behaviour*, 67(5), pp.893–898. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0003347203004597 [Accessed April 14, 2015].

Bonadonna, F. & Bretagnolle, V., 2002. Smelling home: a good solution for burrow-finding in

nocturnal petrels? *The Journal of experimental biology*, 205, pp.2519–2523. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12124375.

Bonadonna, F., Hesters, F. & Jouventin, P., 2003. Scent of a nest: discrimination of own-nest odours in Antarctic prions, *Pachyptila desolata*. *Behavioral Ecology and Sociobiology*, 54, pp.174–178.

Bonadonna, F. & Sanz-Aguilar, A., 2012. Kin recognition and inbreeding avoidance in wild birds: the first evidence for individual kin-related odour recognition. *Animal Behaviour*, 84(3), pp.509–513. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0003347212002758 [Accessed September 24, 2014].

Bonadonna, F., Spaggiari, J. & Weimerskirch, H., 2001. Could osmotaxis explain the ability of blue petrels to return to their burrows at night? *The Journal of experimental biology*, 204, pp.1485–1489.

Borgo, J., 2008. *Effects of olfactory and visual predators on nest success and nest-site selection of waterfowl in North Dakota.* Utah State University. Available at: htp://digitalcommons.usu.edu/etd/43.

Boyd, E.M., 1951. A survey of parasitism of the Starling *Sturnus vulgaris* L. in North America. *The Journal of Parasitology*, 37(1), pp.56–84.

Burger, B. V et al., 2004. Avian exocrine secretions. I. Chemical characterization of the volatile fraction of the uropygial secretion of the green woodhoopoe, *Phoeniculus purpureus*. *Journal of chemical ecology*, 30(8), pp.1603–1611.

Burtt, E.H., 1999. Think Small. *The Auk*, 116(4), pp.878–881.

Caspers, B. a & Krause, E.T., 2011. Odour-based natal nest recognition in the zebra finch (*Taeniopygia guttata*), a colony-breeding songbird. *Biology letters*, 7(2), pp.184–186. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3061170&tool=pmcentrez&rendertype=abstract [Accessed January 21, 2014].

Coppedge, B.R., Talent, L.G. & Engle, D.M., 2007. Effects of Olfactory Attributes and Size of Egg on Rates of Predation of Artificial Ground Nests in Tallgrass Prairie. *The Southwestern Naturalist*, 52(4), pp.453–460. Available at: http://www.bioone.org/doi/abs/10.1894/0038-4909%282007%2952%5B453%3AEOOAAS%5D2.0.CO%3B2.

Elder, W.H., 1954. The oil gland of birds. *The Wilson Bulletin*, 66(1), pp.6–31.

Evans, P.G.H., 1988. Intraspecific nest parasitism in the European starling *Sturnus vulgaris*. *Animal Behaviour*, 36(5), pp.1282–1294. Available at: http://www.sciencedirect.com/science/article/pii/S0003347288801970.

Fauth, P.T., Krementz, D.G. & Hines, J.E., 1991. Ectoparasitism and the role of green nesting material in the European starling. *Oecologia*, 88(1), pp.22–29.

Fluen, T., 2008. *A Comparative Analysis Of Evolutionary Changes In Island Birds*. University of Canterbury, Christchurch, New Zealand.

Gil, D. et al., 2008. Adaptive developmental plasticity in growing nestlings: sibling competition induces differential gape growth. *Proceedings of the Royal Society B: Biological Sciences*, 275(1634), pp.549–54. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2596805&tool=pmcentrez&rendertype=abstract [Accessed November 28, 2015].

Gwinner, H. et al., 2000. Green plants in starling nests: effects on nestlings. *Animal behaviour*, 59(2), pp.301–309. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10675252 [Accessed July 14, 2015].

Haribal, M., Dhondt, A. & Rodriguez, E., 2009. Diversity in chemical compositions of preen gland secretions of tropical birds. *Biochemical Systematics and Ecology*, 37(2), pp.80–90. Available at: http://dx.doi.org/10.1016/j.bse.2008.12.005.

Higgins, P.J., J.M. Peter & S.J. Cowling (eds) 2006. Handbook of Australian, New Zealand and Antarctic Birds. Volume 7: Boatbill to Starlings. Oxford University Press, Melbourne.

Higgins, P.J., J.M. Peter & W.K. Steele (eds) 2001. Handbook of Australian, New Zealand and Antarctic Birds. Volume 5: Tyrant-flycatchers to Chats. Oxford University Press, Melbourne.

Jacob, J., Eigener, U. & Hoppe, U., 1997. The structure of preen gland waxes from pelecaniform birds containing 3,7-dimethyloctan-1-ol - An active ingredient against dermatophytes. *Zeitschrift fur Naturforschung Section C - Journal of Biosciences*, 52(1-2), pp.114–123.

Krause, E.T. & Caspers, B.A., 2012. Are Olfactory Cues Involved in Nest Recognition in Two Social Species of Estrildid Finches? *PLoS ONE*, 7(5), p.e36615. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3344906&tool=pmcentrez&rendertype=abstract [Accessed January 21, 2014].

Lafuma, L., Lambrechts, M.M. & Raymond, M., 2001. Aromatic plants in bird nests as a protection against blood-sucking flying insects? *Behavioural Processes*, 56(2), pp.113–120. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0376635701001917.

Lombardo, M.P. et al., 1989. Egg removal and intraspecific brood parasitism in the European starling (*Sturnus vulgaris*). *Behavioural Ecology and Sociobiology*, 1975(4), pp.217–223.

Martin, T.E., 1993. Nest Predation and Nest Sites. New perspectives on old patterns. *BioScience*, 43(8), pp.523–532.

Martín-Platero, A.M. et al., 2006. Characterization of antimicrobial substances produced by Enterococcus faecalis MRR 10-3, isolated from the uropygial gland of the hoopoe (*Upupa epops*). *Applied and Environmental Microbiology*, 72(6), pp.4245–4249.

Martín-Vivaldi, M. et al., 2010. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. *Proceedings of the Royal Society B: Biological Sciences*, 277, pp.123–130.

Martín-Vivaldi, M. et al., 2009. Seasonal, sexual and developmental differences in hoopoe *Upupa epops* preen gland morphology and secretions: evidence for a role of bacteria. *Journal of Avian Biology*, 40(October 2007), pp.191–205.

Moyer, B.R., Rock, A.N. & Clayton, D.H., 2003. Experimental test of the importance of preen oil in rock doves (Columba livia). *The Auk*, 120(2), pp.490–496.

Pietz, P.J. & Granfors, D.A., 2000. Identifying predators and fates of grassland passerine nests using miniature video cameras. *Journal of Wildlife Management*, 64(1), pp.71–87. Available at: <Go to ISI>://000084785900008.

Price, C.J. & Banks, P.B., 2012. Exploiting olfactory learning in alien rats to protect birds' eggs. *Proceedings of the National Academy of Sciences of the United States of America*, 109(47), pp.19304–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3511072&tool=pmcentrez&rendertype=abstract [Accessed November 28, 2015].

Rajchard, J., 2010. Biologically active substances of bird skin: a review. *Veterinarni Medicina*, 55(9), pp.413–421. Available at: <Go to ISI>://000282708500001.

Reneerkens, J. et al., 2008. Seasonally changing preen-wax composition: red Knots' (*Calidris canutus*) Flexible Defense Against Feather-Degrading Bacteria? *The Auk*, 125(2), pp.285–290.

Reneerkens, J., Piersma, T. & Damsté, J.S.S., 2005. Switch to diester preen waxes may reduce avian nest predation by mammalian predators using olfactory cues. *The Journal of experimental biology*, 208, pp.4199–4202.

Richner, H., Oppliger, A. & Christe, P., 1993. Effect of an ectoparasite on reproduction in great tits. *Journal of Animal Ecology*, 62, pp.703–710.

Ricklefs, R.E., 1969. An analysis of nesting mortality in birds. *Smithsonian Contributions to Zoology*, (9), pp.1–48.

Scott-Baumann, J.F. & Morgan, E.R., 2015. A review of the nest protection hypothesis: does inclusion of fresh green plant material in birds' nests reduce parasite infestation? *Parasitology*, 142(8), pp.1016–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25804728 [Accessed November 29, 2015].

Shawkey, M.D., Pillai, S.R. & Hill, G.E., 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. *Journal of Avian Biology*, 34(4), pp.345–349.

Soini, H.A. et al., 2013. Chemosignaling diversity in songbirds : Chromatographic profiling of preen oil volatiles in different species. *Journal of Chromatography A*, 1317, pp.186–192. Available at: http://dx.doi.org/10.1016/j.chroma.2013.08.006.

Soler, J.J. et al., 2008. Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. *Functional Ecology*, 22(5), pp.864–871.

Sweeney, R.J., Lovette, I.J. & Harvey, E.L., 2004. Evolutionary variation in feather waxes of passerine birds. *Auk*, 121(2), pp.435–445. Available at: http://search.epnet.com/login.aspx?direct=true&db=aph&an=13377085.

Thierry, A., 2014. *Predation risk and the evolution of odours in island birds*. University of Canterbury, Christchurch, New Zealand.

Tschirren, B., Fitze, P.S. & Richner, H., 2003. Sexual dimorphism in susceptability to parasites and cell-mediated immunity in great tit nestlings. *Journal of Animal Ecology*, 72, pp.839–845.

Whelan, C.J. et al., 1994. Effects of Olfactory Cues on Artificial-Nest Experiments. *The Auk*, 111(4), pp.945–952.

Zhang, Y.-H., Du, Y.-F. & Zhang, J.-X., 2013. Uropygial gland volatiles facilitate species recognition between two sympatric sibling bird species. *Behavioral Ecology*, 24(6), pp.1271–1278. Available at: http://www.beheco.oxfordjournals.org/cgi/doi/10.1093/beheco/art068 [Accessed March 5, 2015].

Chapter 3

An experimental test of the effect of diet on preen wax composition in New Zealand silvereyes (*Zosterops lateralis*)*

***Note this chapter has been published as:**

Azzani, L., Rasmussen, J.L., Gieseg, S. and Briskie, J.V. (2016). An experimental test of the effect of diet on preen wax composition in New Zealand silvereyes (*Zosterops lateralis*). Chapter 33 in: Chemical Signals in Vertebrates 13 (B.A. Schulte, T.E. Goodwin, M.H. Ferkin, eds). Springer, Basel.

Abstract: *Most birds have a uropygial gland, which produces preen wax that birds spread over their feathers when preening. Preen waxes contain a variety of volatile components which may function, in some species, as signals to communicate identity of mates and nest sites, or provide information about the owner's state. Such signals may not be reliable if preen wax composition is readily altered by changes in diet. We used a diet supplementation experiment in a New Zealand population of silvereyes, Zosterops lateralis, to determine if preen wax composition varied with diet. During the austral breeding season, free-living silvereyes were fed a mix of animal tallow ad libitum for a period of 22 days. Their preen waxes were collected 6 and 7 days (sampling period 1), 11 days (sampling period 2), and 21 and 22 days (sampling period 3) after the beginning of the diet supplementation to determine if preen wax composition would change in response to the addition of the supplemental food. Birds sampled within a 7-day period leading up to the diet supplementation acted as controls. We found no significant changes in the preen wax throughout the study period. Feeding did not affect preen wax composition, and there was no change in preen wax composition among sampling periods. Date of sampling also did not affect preen wax composition during the period of the experiment. As the preen wax of silvereyes is known to change between non-breeding and breeding seasons, the lack of change in our study was not simply the result of preen wax production being fixed. Instead, our results support the hypothesis that preen wax composition in silvereyes is determined by endogenous mechanisms and is not affected by day to day variation in diet.*

Introduction

Research in avian communication has increasingly focused on “odour signatures”, defined as olfactory or chemical cues that enable individual recognition (see Bonadonna and Mardon 2013, Caro et al. 2015 for a review). Odour signatures, and olfactory signals in general, could function in territorial marking and defence (Rich and Hurst 1998; Hayward and Hayward 2010), individual recognition (Mardon et al. 2010) and mate choice (Balthazart and Schoffeniels 1979; Hirao et al. 2009). However, for odour signatures to function in communication, they need to be consistent (or at least predictable) over time within the same individual (Hauser 1997). Kwak et al. (2008) point out that individual characteristics (not just individual chemical signatures) are assumed to be relatively invariant over time if they function as a cue for individual recognition, and this stability would therefore be expected of olfactory signatures. Nevertheless, short-term fluctuations in body odour due to stress, diet and individual condition are known (Valenta and Rigby 1968; Ferkin et al. 1997; Yamazaki et al. 2002).

A signature role for odours would be less likely if odours were easily, and directly, modified by environmental factors, such as changes in diet. If the odour profile of an animal closely reflects what it just ate rather than some intrinsic, individual characteristic, then communication of individual qualities based upon it could be impaired. For example, Kwak et al. (2008) demonstrated that mice were able to detect a change in diet of congeners by odour alone more readily than a difference in MHC-dependent odours. Nevertheless, variation in diet did not mask the expression of MHC-dependent odour types, as suggested by previous studies (Brown et al. 1996; Schellinck et al. 1997). Despite the increasing realisation that odours may play an important role in social communication in birds (Caro and Balthazart 2010), the influence of diet on odours in birds, but also vertebrates in general, has been tested in only a few studies (non-avian vertebrates: Ferkin et al. 1997; Havlicek & Lenochova 2006; Kwak et al. 2008; Osada et al. 2011).

Most studies on the influence of diet on odour production in birds have been carried out by poultry researchers, aimed at investigating differences in taste and smell of meat and eggs produced by broilers fed different diets (Mielnik et al. 2002; Overland and Skrede 2012). Dietary lipids are readily assimilated and stored in fat deposits in birds, as well as influencing fatty acid composition of other tissues and organs (McCue et al. 2009), and so it is not surprising that researchers have confirmed a direct link between food intake and meat fat quality (Bou et al. 2005; Shin et al. 2011). However, the main source of odours in most birds are the preen waxes produced by the uropygial gland (Jacob et al. 1979; Mardon et al. 2010; Caro et al. 2014), which are likely to have a different physiological mechanism of production than that of body fat (Apandi and Edwards 1964;

Sandilands et al. 2004). The uropygial gland has a high capacity for synthesizing lipids *de novo* (Noble et al. 1963; Tang and Hansen 1972; Buckner and Kolattukudy 1975; Jacob and Ziswiler 1982; Stevens 1996). However, the composition of preen wax is known to change with season in some species (Reneerkens et al. 2002), and it is possible that such changes could be at least partly triggered by concurrent seasonal changes in diet. Given that hormonal changes also occur seasonally, experiments that manipulate food intake independently of season are needed to determine whether diet alone affects changes in preen wax composition.

To date, the results of experimental tests of diet on preen wax composition have been variable. Thomas et al. (2010) found that diet affected preen wax composition in a feeding study on white-throated sparrows, *Zonotrichia albicollis*. The treatment consisted of an artificial diet enriched with sesame oil or fish oil; these oils contain fatty acids that differ in their average chain length and in their n3:n6 ratio. The authors found that birds fed the sesame oil diet had longer chain monoesters than those fed the fish oil diet. Apandi and Edwards (1964) found a similar effect of corn-oil supplementation on the composition of uropygial secretions of chickens. In contrast, Pan et al. (1979) found no effect of diet on preen wax composition in domestic poultry. An experimental study on the "make-up hypothesis" in tawny owls, *Strix aluco*, also indirectly suggests no effect of diet on preen wax composition, at least with respect to uropygial compounds that contribute to bill reflectance (Piault et al. 2008). In some species of sandpipers, one food-restriction experiment showed an effect of diet (Reneerkens et al. 2007), but unpublished results reported in the same paper suggest an absence of dietary effects on the composition of uropygial gland secretions (reported in Reneerkens et al. 2006). In short, there have been few experiments designed to test the influence of diet on preen wax composition. Even if the biochemical pathways between dietary intake and preen wax production are not direct, changes in energy budgets as a result of dietary changes could affect preen wax production.

Determining if the odours of wild birds are influenced by changes in diet would help explain differences in odours of birds occupying different habitats (Levy and Strain 1982) and the seasonal changes in preen wax composition reported for some birds (Reneerkens et al. 2002), which may or may not be partly influenced by seasonal changes in diet (Shaw et al. 2011). Alternatively, confirming that diet has little role in preen wax composition, would support a greater role for a genetic basis to odours (as shown in mice by Kwak et al. 2008), which may be used to reliably signal information such as gender or individual identity. To this end, we carried out a diet supplementation experiment on a wild population of silvereyes, *Zosterops lateralis*, in New Zealand. As progression in the breeding season and sex can affect preen wax composition in a number of species (Caro and Balthazart 2010), we also examined the effect of sampling date and

sex on preen wax composition.

Materials and methods

Study site and species

The experiment took place in a regenerating native forest on the east coast of the South Island, New Zealand (Kowhai Bush Reserve, 173°36'E, 42°23'S). The silvereye is a small (11-14 g) passerine bird that ranges throughout Australia and the South Pacific, although it only recently self-colonised New Zealand, with the first birds arriving in the early 19th century (Higgins et al. 2006). They have a generalist diet, feeding on a variety of insects, fruit and nectar (review in Higgins et al. 2006), although there have been no detailed studies of diet of silvereyes at the study site. Sexes are similar in size (mass(g): females = 12.9± 1.1, males= 12.4± 1.2) and coloration, and both parents perform incubation and feeding duties. From 22 November to 16 December 2011 birds were captured using mist nets (under banding permit 2008078 and research permit NM-34075-FAU). This coincides with the period of peak nesting for this species at the study site. Each bird was handled using a new pair of latex gloves and placed in a clean paper bag before processing. This precaution was taken to minimize contamination of the preen wax samples with oil from the hands of the handlers. Preen wax samples were collected by gently squeezing the uropygial gland with forceps with wax-coated tips; the extruded droplet was then collected on a clean stainless-steel inoculation loop and placed in a clean glass insert lodged inside a glass vial, sealed with teflon-covered lids to minimize evaporation or contamination of samples. Vials were kept cool using freezer packs in the field for a maximum of 8 hours, until later frozen. Samples were kept at -20°C until analysis. All birds (n=107) were banded with numbered aluminium bands to allow resampling of individuals as the season and experiment progressed.

Diet supplementation experiment

Birds were given *ad libitum* access to tallow (a commercially available mixture of drippings from beef, mutton and pork fat; “Butchery Pure Dripping” brand) for 22 days, from 26 November to 17 December 2011. The fat was provided in a series of feeders set up in the study area. Silvereyes readily fed on the fat, and earlier work had confirmed that supplemental feeding significantly increased reserves of body fat in this species (Barnett and Briskie 2007). Consumption of fat was monitored to ensure the feeders were always stocked. No birds were caught or sampled during the first 5 days of the feeding experiment to allow time for the changes in the energy balance of silvereyes to influence their preen wax production. The time needed for diet to start affecting preen

wax composition is unknown but a period of 5 days was deemed to be sufficient as the time-frame was comparable to that employed in one other study (Piault et al. 2008) and the feeding protocol used is known to change both the fat reserves and singing behaviour of silvereyes within a 24 hour period (Barnett and Briskie 2007). Sampling of birds occurred on the 6th and 7th day after the feeders were deployed (1 and 2 December 2011; sampling period 1). Birds were sampled again 11 days (6 December 2011; sampling period 2) and 21 and 22 days (16 and 17 December 2011; sampling period 3) after the feeders were deployed. In addition to these 3 time points, "pre-feeding" samples were also collected from birds caught within the 7-day period preceding the commencement of diet supplementation (no birds could be collected from 20 to 22 November because of adverse weather conditions, so the actual time-frame for the pre-feeding samples was 4 days). The temporal structure of the experiment was chosen to allow for the detection of any changes in preen wax composition that may be related to a change in diet and energy intake of silvereyes.

Sample preparation and analysis

Samples were dissolved in 100 µl of ethyl acetate, poured directly into the insert containing the inoculation loop and the preen wax. The vial was then re-capped, and vortexed for 60 seconds at 700 rpm to ensure dissolution of the preen wax. The original cap was then substituted with a chromatographic cap fitted with a single-use PTFE silicone septum. Samples were analysed on a Shimadzu GC-2010 gas chromatograph, equipped with a Shimadzu AOC-20i+s auto-injector and a Varian CP-SIL 5 CB capillary column (25 m length x 320 µm internal diameter x 0.12 µm film thickness). Injection volume was 1 µl, with a 6:1 split ratio. Injection port temperature was set at 250°C, the carrier gas was nitrogen with a total flow of 19.0 ml/min and a linear velocity of 36.7 cm/sec. The FID detector operated at 320°C, with a sampling interval of 40 msec. Oven temperature was programmed as follows: initial temperature 70°C with a hold time of 4 mins, then increased to 130°C at a rate of 20°C/min, and finally increased to 320°C at 4°C/min rate (hold time 15 mins). Results were recorded on Shimadzu's GCSolution, version 2.3 (©Shimadzu 2002-2009) software.

Quality control

Sequences of linear alkanes ranging from C6 to C40 (C7-C40 Saturated Alkane Mixture in hexane, 49452-U Supelco, Sigma-Aldrich), and palmytoil palmytate (C16-C16 ester) were injected at regular intervals during the analysis period to ensure consistency and monitor column performance. In addition, empty vials, that were manipulated in the field and processed in the lab in the same manner as sample vials, were analysed to control for any background or environmental odours.

Post-processing

Traces with an absolute maximum intensity in the peak region inferior to 8000 uV were excluded from further analysis. Low trace intensity was most likely due to an insufficient quantity of preen wax having been drawn from the bird. As it was hard to standardize the quantity of preen wax extracted from each bird, we used the relative proportions of compounds rather than their absolute quantities. Observations were post-processed in R (R Core Team 2014), by aligning the retention times and areas for each peak across all observations. Peaks that were present on a chromatogram but fell below the detection limit of the instrument were given an arbitrary value of 0.000001. Only peaks that were above the detection limit for the instrument in at least 5% of the samples were included in the analysis. The area of each peak was converted to its proportional contribution to total peak area in that sample. The proportions were then square-root transformed, to reduce the influence of large peaks (Borcard et al. 2011). These two steps correspond to the Hellinger transformation, and the Euclidean distance calculated on transformed data is identical to the Hellinger distance (Legendre and Birks 2012). This distance is metric and has proven efficient in separation of ecological datasets (Legendre and Gallagher 2001; Anderson and Willis 2003; Kindt and Coe 2005)

Statistical analysis

Differences in the chemical profiles of birds among the different diet time points (encoded in a matrix of Hellinger distances) were analysed using two multivariate techniques: (1) permutational MANOVA (“non parametric MANOVA”, after Anderson 2001, implemented in R, package vegan (Oksanen et al. 2013), function adonis) and (2) Canonical Analysis of Principal Coordinates (CAP, Anderson & Willis 2003, implemented via FORTRAN program by M.J. Anderson). These two techniques share some similarities, but each is expected to perform slightly better in different situations, depending on the data structure and the correlation matrix of the dependent variables; PERMANOVA is expected to perform better when the dependent variables are not highly correlated, while for a dataset containing several abundant variables that are highly correlated, CAP can be more efficient at detecting significant changes in minor variables that are not correlated with the former (Anderson 2004). Adonis allows testing of multi-way hypotheses, whereas CAP, as implemented in FORTRAN, only allows to test one grouping factor at a time, so the joint effect of “diet treatment” and “sex” was analysed in Adonis only. Both PERMANOVA and CAP possess some key advantages over other multivariate techniques: they allow any dissimilarity measure to be used, rather than being limited to metric distance measures, and, they do not require the response variables to meet stringent assumptions, such as multivariate normality, which are seldom satisfied

in chemoecological data sets (Anderson and Willis 2003). Finally, CAP enables us to generate a visual representation of the constrained ordination (by plotting samples against axes that maximise the differences between the specified *a priori* groups, i.e. the canonical axes), which can be compared with a robust unconstrained ordination (i.e. NMDS) to explore the multivariate patterns in the dataset (Anderson and Willis 2003).

Effect of sampling date on preen wax composition was evaluated using both multivariate and univariate techniques. Because of the temporal structure of the diet supplementation experiment, and because data belonging to three of the four time points (i.e., pre-diet supplementation, sampling period 1, sampling period 3) were collected over a period of days, we also performed a PERMANOVA analysis, on the same Hellinger distances, to verify whether collection date, rather than feeding time point, was correlated with variation in chemical distances.

Recaptured birds were excluded from the above analyses (n=92) to avoid pseudoreplication: the samples used in the above analysis have all been collected from different individuals (Pre feeding: n=9; 6 days feeding: n=22; 11 days feeding: n=26; 21 days feeding: n=35). The number of samples in the pre-feeding group is rather low but, while a multivariate version of power analysis could not be performed, I checked for sample size adequacy following the method outlined by Anderson and Santana-Garcon (2015), and found it to be satisfactory (results not shown).

Moreover, in the context of chemical profiles being used as individual signature, it is interesting to investigate the "consistency" of such signals (i.e. if individuals maintain a coherent signature in the face of dietary changes). To this end, we performed a separate analysis – Response of individual birds to food supplementation – testing for effect of diet on individual birds that were captured during more than one sampling period. Because of a smaller sample size (n=15 different individuals), we split the 30 replicate samples into two groups: "sampling period 1", comprised of birds first captured 5 days after the beginning of diet supplementation, and "later sampling periods", comprised of the same individuals recaptured 5 or 14 to 16 days later. To analyse multivariate responses from repeated samples, PERMANOVA was carried out using vegan function adonis to obtain a value for the model pseudo-F ratio. Then, specifying a custom permutation scheme to take into account dependency of data points, a "trustworthy" P-value was obtained via a randomization test, with 1999 permutations restricted within individuals.

Results

Effect of diet treatment and sex

Clustering based on the diet treatment did not appear to be supported either in the NMDS or in the

CAP ordination (Figures 3.1a and 3.1b). CAP analysis did not highlight any significant differences among pre-diet supplementation and the three diet time points. The squared correlations of the three canonical axes extracted from 7 PCO (δ^2_1 = 0.211, δ^2_2= 0.082 and δ^2_3 = 0.025) were not significantly higher than correlations obtained by random permutation of the observations (number of permutations = 9999, trace statistic = 0.319, P = 0.10). 'Leave-one-out allocation' of observations to pre-specified groups resulted in 36% of cases being correctly classified, which is similar to the proportion of observations correctly classified by chance alone, i.e. 28% (for details of this calculation, see Tabachnick and Fidell 2006, p. 404). A two-way PERMANOVA also yielded non significant results for both feeding treatment (Pseudo-$F_{3,84}$ = 1.47, P = 0.082) and sex (Pseudo-$F_{1,84}$ = 1.37, P = 0.19). Sex*Treatment interaction was tested in a previous model, found to be non significant (P = 0.97), and therefore removed from the final model. Thus, there was no evidence that food supplementation or sex affected the preen wax profiles of silvereyes.

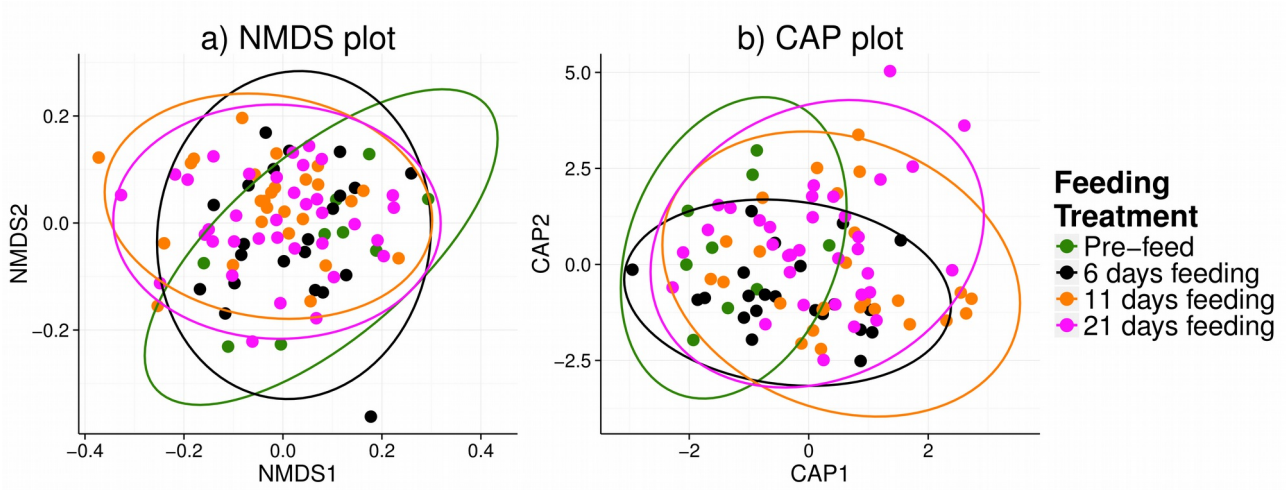

Figure 3.1: a) NMDS plot from unconstrained ordination and b) CAP plot from constrained analysis of preen wax profiles' from independent samples collected during the feeding experiment. Each point corresponds to one individual, i.e. one chemical profile.

Effect of collection date

As our experiment was carried out over a period of several weeks, seasonal changes in preen wax composition may have affected the results. To determine if seasonal changes were apparent in our study, we examined the effect of collection date on preen wax composition. No change with date was detected during the course of the experiment. Canonical Correlation Analysis of multivariate profiles correlation with sampling date was not significant (One canonical axis extracted from 4 PCO, δ^2_1 = 0.079, trace statistic = 0.079, P = 0.12). It is important to note that the number of PCO upon which to draw the axis is chosen in this case to minimize the residual sum of squares, rather than to maximize the proportion of correctly classified observations (i.e., using a continuous

variable rather than a grouping factor to classify against). PERMANOVA on the same dataset also did not reveal any significant differences (Pseudo-$F_{1,90}$ = 1.47, P = 0.16). Both analyses suggest date had no effect on the profiles of preen wax over the course of the supplementation experiment.

Response of individual birds to food supplementation

Birds sampled during feeding period 1 and recaptured during feeding period 2 or 3 did not exhibit any significant change in preen wax profile (Repeated measures PERMANOVA: Pseudo-$F_{1,28}$ = 0.68, P = 0.36). Preen wax profiles did not appear to mirror the duration of diet supplementation: the preen wax profiles were either unchanged (see example in Figure 3.2) or the change was not unidirectional. This lack of any consistent change between sampling periods suggests the feeding regime was an unlikely explanation for such diffused changes (Figure 3.3). In contrast, two artificial datasets produced by simulating a big or small effect of feeding on the preen wax profile resulted in both a significant repeated measures PERMANOVA and a NMDS plot that clearly highlighted the clustering of samples based on feeding status (see online supplementary material).

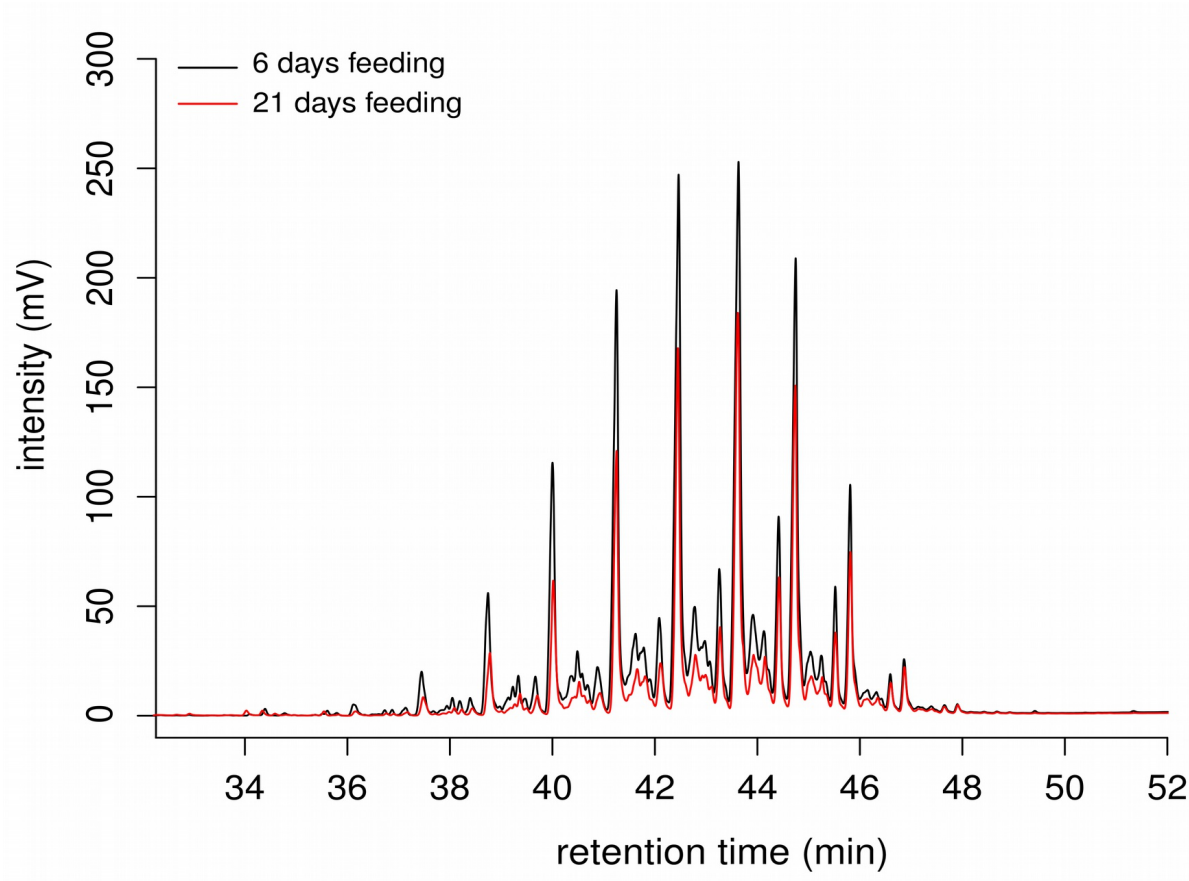

Figure 3.2: Example of a silvereye's chemical profile. For graphical clarity, only a subsection of the chromatogram is displayed, centered around the ester section of the preen wax (Rt: 32-52 minutes). Pictured here are chromatograms obtained from a recaptured individual (SE50): the profile remains substantially unchanged.

Discussion

Dietary fat supplementation did not affect preen wax composition in silvereyes, both in the population as a whole and within individuals. The remarkable consistency of chemical profiles of silvereyes in the face of sizeable changes in dietary intake would suggest preen wax composition is largely controlled endogenously, and thus our study supports the hypothesis that preen wax in silvereyes would qualify as a state signal that carries information about the physiological and genetic attributes of an individual (Bonadonna and Mardon 2013).

As direct incorporation of fat from food into the preen wax seems unlikely (Noble et al. 1963; Buckner and Kolattukudy 1975), it is still possible that changes in energetic intake could stimulate hormonal synthesis or release which would in turn influence the activity of the uropygial gland. The uropygial gland is particularly rich in receptors for steroid hormones and the activity of the gland is known to be under the influence of hormones (Asnani and Ramachandran 1993; Whittaker et al. 2011). Furthermore, the enzymes responsible for fatty acid synthesis and modification are genetically determined, and their activity is endogenously regulated (Bohnet et al. 1991; Kolattukudy et al. 1991). The absence of an effect in our study could indicate that the diet supplementation period was too short for such hormonal modifications to be instated. However, steroid-induced stimulation is known to act within hours in humans (Falkenstein et al. 2000), and acute steroidogenesis, which could be enhanced by increased dietary fats, occurs in a matter of hours (Miller and Bose 2011). The timing of our experiment, therefore, seems to be long enough to allow detection of such indirect effects. Given that our feeding protocol is known to change both the fat reserves and singing behaviour of silvereyes within a 24 hour period (Barnett and Briskie 2007), it also seems unlikely that we failed to induce any change in preen wax because of inadequate exposure to the treatment.

Another possible explanation for the failure of our experiment to change preen wax composition is that physiological pathways of preen wax production are completely fixed. However, preen wax composition in silvereyes is known to vary seasonally, changing between non-breeding and breeding seasons (Fluen (2008), *our unpublished data*). This is a pattern found in many species of birds and typically involves a decrease in the production of more volatile monoesters (and increase of less volatile diesters) when individuals are nesting, perhaps as an adaptation to camouflage nests from predators that use olfaction to locate their prey (Reneerkens et al. 2005). Seasonal changes in the preen wax of silvereyes indicates that preen wax production is not completely fixed, but instead that any changes are presumably the result of endogenous mechanisms (e.g. hormonal states) accompanying breeding, and not local changes in the type and

quantity of food available.

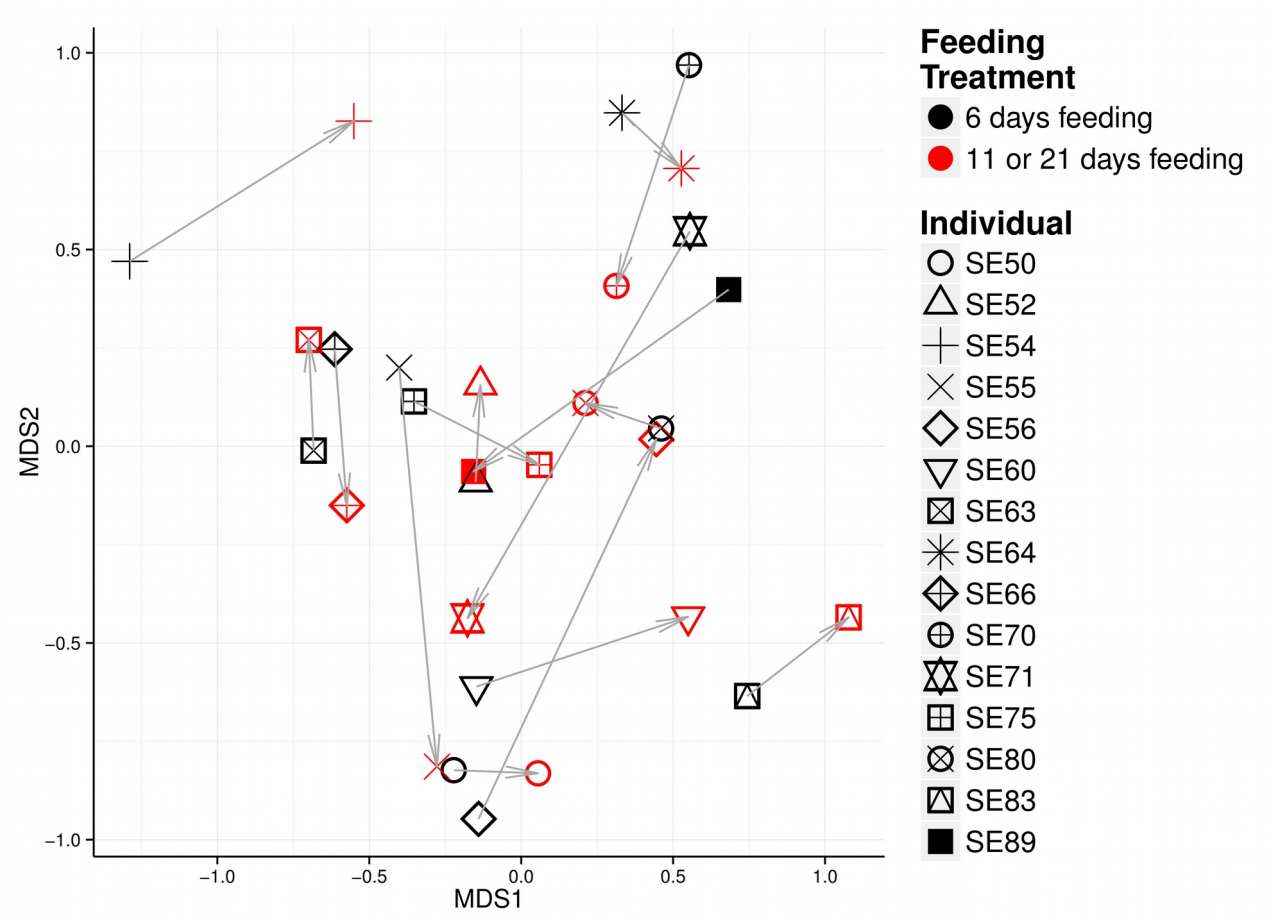

Figure 3.3: NMDS plot, multivariate intra-individual (repeated measures) comparison: lines connect preen wax profiles from 15 individuals sampled in period 1 (black) and periods 2 and 3 (red).

Although we did not find any significant change in preen wax composition in silvereyes, it is possible that providing supplemental food may have increased the amount or volume of wax produced per unit time. An increase in the rate of preen wax production could then be used as a signal, with those individuals producing more wax (and presumably more persistent odour signals), conveying their better condition, in a manner analogous to studies showing high quality individuals having more colourful plumages, or more complex song repertoires (e.g. Hill 1991). Unfortunately, it was not feasible to reliably measure the volume of preen wax production in silvereyes in our experiment.

The lack of a change in preen wax with diet is consistent with the hypothesis that such compounds could be used as reliable chemosignals. Previous workers have found evidence that chemosignals are used by birds for species recognition (Bonadonna and Mardon 2010; Zhang et al. 2013, Krause et al. 2014), for kin recognition (Coffin et al. 2011, Krause et al. 2012), and for

homing to the nest (Bonadonna and Bretagnolle 2002; Krause and Caspers 2012). All functions require a relatively stable odour profile, that can (a) reliably convey information about the genetic make-up of an individual or (b) be recognizable over time in order to be used as an effective cue for locating and returning to the nest. Equally, if avian chemosignals are to function as cues for reproduction, (i.e. convey information about the sexual maturity and/or sexual receptiveness of an individual) (Bohnet et al. 1991; Hirao et al. 2009), the chemical profile has to be robust to transient changes such as those introduced by diet. Nevertheless, at present it is not known if the odours produced by preen wax are used by silvereyes in communication. There are no sexual differences in preen wax composition between males and females, as is found in other species. It bears noticing that the similarity in preen wax composition between the sexes in silvereyes could be related to the fact that both males and females share in incubation and brooding. However, allopreening behaviour is common in silvereyes and occurs regularly between members of a pair, between siblings in a nest, and between parents and offspring (Kikkawa and Wilson 1983). Allopreening puts individuals in direct contact with the preen wax (and thus odours) of other individuals, and thus has the potential to be used for individual recognition and perhaps measures of quality.

The extent to which birds use chemical signals is species-specific, possibly related to the ecology of a species and to the greater or lesser extent to which it can use other senses (Martin 2012). Comparative studies have only been undertaken within Procellariiformes (Bonadonna and Bretagnolle 2002; Cunningham 2003), but other species also use olfactory cues for predator detection (Amo et al. 2011) although this ability is not universal (Johnson et al. 2011). It is possible that species which use endogenous olfactory cues as state signals show little modification of their profile in response to diet (for the reasons stated above), whereas species that do not rely on a signalling function could have a less “stable” profile, but there are too few studies at present to examine this hypothesis.

Our experiment only manipulated one aspect of the silvereye diet (i.e. fat intake) and we cannot rule out that other changes to diet might induce compositional changes in preen wax. Fat was chosen because of the effect it has on body mass and fat reserves, as well as on dawn chorus performance (Barnett and Briskie 2007) and breeding behaviour in silvereyes (Barnett and Briskie 2010). It would be worthwhile manipulating the diet of silvereyes in other ways to determine whether the lack of change we observed is a general pattern across all types of diets. Lab studies have the advantage in that diet can be manipulated under controlled conditions as our use of wild birds meant it was not possible to control an individual’s entire diet. However, field experiments have the undoubted value of mirroring what happens under natural diet variations. As our study is the first to determine whether diet can alter preen wax in a wild bird, further trials are needed to

confirm that any diet-induced changes in the lab are likely to occur in the wild.

Finally, despite the fact that we did not detect any significant change in the profiles of preen wax in relation to either sex, date or diet supplementation, does not mean preen wax composition is identical between individuals. Both the analyses on independent samples and on recaptured birds showed that there is remarkable variation among individuals. Our results indicate this variation is not correlated to diet, sex or seasonality. However, our results do not reveal the factors responsible for the differences in preen wax composition among individuals. The next step is to determine whether the variation observed in chemometric analyses of preen wax can be perceived by the birds, and if so, what role this variation plays in either intra- or inter- specific communication.

Acknowledgements

The authors wish to thank David Lloyd-Jones and Matt Weiland for help in the field, Jack Van Berkel, Ngaire Button and the Kaikoura Field Station for providing accommodation and research space during our field season, and the Royal Society of New Zealand Marsden Fund for funding the project. L.A. Would also like to acknowledge the contribution of Kay Cichini, who wrote the code to implement the repeated measures PERMANOVA and made it publicly available on his blog. The original code can be found at http://thebiobucket.blogspot.co.nz/2011/04/repeat-measure-adonis-lately-i-had-to.html

Supplementary material

The file “abundance_data.txt” contains the data used for the analysis in section “Response of individual birds to food supplementation”. The file “simulated_rep_measures_analysis.R” is an R script containing the code necessary to run a repeated measures PERMANOVA such as the one presented in the section above. When running this script, however, our original abundance matrix is modified to simulate an effect of treatment (with two different magnitudes), in order to test whether the technique we used is able to detect such an effect, i.e. to return a significant result. The p-values and ordination plots output from this script can be compared to the ones in section “Response of individual birds to food supplementation”.

References

Amo L, Visser ME, Oers K van (2011) Smelling Out Predators is Innate in Birds. Ardea 99:177–184. doi: 10.5253/078.099.0207

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecol 26:32–46.

Anderson MJ (2004) Canonical Analysis of Principal coordinates. A computer program by Marti J. Anderson. Department of Statistics, University of Auckland.

Anderson MJ, Willis TJ (2003) Canonical Analysis of Principal Coordinates: a Useful Method of Constrained Ordination for Ecology. Ecology 84:511–525.

Apandi M, Edwards HM (1964) Studies on the Composition of the Secretions of the Uropygial Gland of Some Avian Species. Poult Sci 43:1445–1462.

Asnani M V, Ramachandran A V (1993) Roles of Adrenal and Gonadal Steroids and Season in Uropygial Gland Function in Male Pigeons, *Columba livia*. Gen Comp Endocrinol 92:213–224. doi: http://dx.doi.org/10.1006/gcen.1993.1157

Balthazart J, Schoffeniels E (1979) Pheromones Are Involved in the Control of Sexual Behaviour in Birds. Naturwissenschaften 66:55–56.

Barnett CA, Briskie J V (2007) Energetic state and the performance of dawn chorus in silvereyes (*Zosterops lateralis*). Behav Ecol Sociobiol 61:579–587. doi: 10.1007/s00265-006-0286-x

Barnett CA, Briskie J V (2010) Silvereyes *Zosterops lateralis* increase incubation attentiveness in response to increased food availability. Ibis (Lond 1859) 152:169–172.

Bohnet S, Rogers L, Sasaki G, Kolattukudy PE (1991) Estradiol induces proliferation of peroxisome-like microbodies and the production of 3-hydroxy fatty acid diesters, the female pheromones, in the uropygial glands of male and female mallards. J Biol Chem 266:9795–9804.

Bonadonna F, Bretagnolle V (2002) Smelling home: a good solution for burrow-finding in nocturnal petrels? J Exp Biol 205:2519–2523.

Bonadonna F, Mardon J (2013) Besides Colours and Songs, Odour is the New Black of Avian Communication. In: East ML, Dehnhard M (eds) Chem. Signals Vertebr. 12. Springer New York, pp 325–339

Bonadonna F, Mardon J (2010) One House Two Families: Petrel Squatters Get a Sniff of Low-Cost Breeding Opportunities. Ethology 116:176–182. doi: 10.1111/j.1439-0310.2009.01725.x

Borcard D, Gillet F, Legendre P (2011) Numerical Ecology with R. Springer, New York

Bou R, Guardiola F, Barroeta a C, Codony R (2005) Effect of dietary fat sources and zinc and selenium supplements on the composition and consumer acceptability of chicken meat. Poult Sci 84:1129–1140.

Brown RE, Schellinck HM, West AM (1996) The influence of dietary and genetic cues on the ability of rats to discriminate between the urinary odors of MHC-congenic mice. Physiol Behav 60:365–372. doi: 10.1016/0031-9384(96)00030-3

Buckner JS, Kolattukudy PE (1975) Lipid biosynthesis in the sebaceous glands: synthesis of multibranched fatty acids from methylmalonyl-coenzyme A in cell-free preparations from the uropygial gland of goose. Biochemistry 14:1774–1782.

Caro SP, Balthazart J (2010) Pheromones in birds: Myth or reality? J Comp Physiol A Neuroethol Sensory, Neural, Behav Physiol 196:751–766. doi: 10.1007/s00359-010-0534-4

Caro SP, Balthazart J, Bonadonna F (2015) The perfume of reproduction in birds: chemosignaling in avian social life. Horm Behav 68:25–42. doi: 10.1016/j.yhbeh.2014.06.001

Coffin HR, Watters J V., Mateo JM (2011) Odor-based recognition of familiar and related conspecifics: A first test conducted on captive Humboldt penguins (*Spheniscus humboldti*). PLoS One 6:10–13. doi: 10.1371/journal.pone.0025002

Cunningham GB (2003) A comparison of the olfactory abilities of three species of procellariiform chicks. J Exp Biol 206:1615–1620. doi: 10.1242/jeb.00286

Falkenstein E, Tillmann HC, Christ M, et al (2000) Multiple actions of steroid hormones - a focus on rapid, nongenomic effects. Pharmacol Rev 52:513–56.

Ferkin MH, Sorokin ES, Johnston RE, Lee CJ (1997) Attractiveness of scents varies with protein content of the diet in meadow voles. Anim Behav 53:133–141. doi: 10.1006/anbe.1996.0284

Hauser MD (1997) The evolution of communication. MIT press

Havlicek J, Lenochova P (2006) The effect of meat consumption on body odor attractiveness. Chem Senses 31:747–752. doi: 10.1093/chemse/bjl017

Hayward G, Hayward M (2010) Potential amplification of territorial advertisement markings by black-backed jackals (*Canis mesomelas*). Behaviour 147:979–992. doi: 10.1163/000579510X499434

Higgins, PJ, Peter JM, Cowling SJ (2006) Handbook of Australian, New Zealand and Antarctic Birds. Volume 7: Boatbill to Starlings. Oxford University Press, Melbourne. Pages 1745-1792

Hill GE (1991) Plumage coloration is a sexually selected indicator of male quality. Nature 350:337–339.

Hirao A, Aoyama M, Sugita S (2009) The role of uropygial gland on sexual behavior in domestic chicken *Gallus gallus domesticus*. 80:115–120. doi: 10.1016/j.beproc.2008.10.006

Jacob J, Balthazart J, Schoffeniels E (1979) Sex Differences in the Chemical Composition of Uropygial Gland Waxes in Domestic Ducks. Biochem Syst Ecol 7:149–153.

Jacob J, Ziswiler V (1982) Chapter 4 - THE UROPYGIAL GLAND. In: Farner D, King J, Parkes K (eds) Avian Biol. Academic Press, Amsterdam, pp 199–324

Johnson LS, Murphy SM, Parrish GW (2011) Lack of predator-odor detection and avoidancec by a songbird, the house wren. J F Ornithol 82:150–157. doi: 10.1111/j.1557-9263.2011.00317.x

Kikkawa J, Wilson JM (1983) Breeding and dominance among the Heron Island silvereyes *Zosterops lateralis chlorocephala*. Emu 83:181–198.

Kindt R, Coe R (2005) Tree Diversity Analysis: A Manual and Software for Common Statistical Methods for Ecological and Biodiversity Studies. World Agrofirestry Centre

Kolattukudy PE, Bohnet S, Sasaki G, Rogers L (1991) Developmental changes in the expression of S-acyl fatty acid synthase thioesterase gene and lipid composition in the uropygial gland of mallard ducks (*Anas platyrhynchos*). Arch Biochem Biophys 284:201–206. doi: http://dx.doi.org/10.1016/0003-9861(91)90284-P

Krause ET, Brummel C, Kohlwey S, et al (2014) Differences in olfactory species recognition in the females of two Australian songbird species. Behav Ecol Sociobiol 68:1819–1827. doi: 10.1007/s00265-014-1791-y

Krause ET, Caspers BA (2012) Are Olfactory Cues Involved in Nest Recognition in Two Social Species of Estrildid Finches? PLoS One 7:e36615. doi: 10.1371/journal.pone.0036615

Krause ET, Krüger O, Kohlmeier P, Caspers BA (2012) Olfactory kin recognition in a songbird. Biol Lett 8:327–329. doi: 10.1098/rsbl.2011.1093

Kwak J, Willse A, Matsumura K, et al (2008) Genetically-based olfactory signatures persist despite dietary variation. PLoS One 3:e3591. doi: 10.1371/journal.pone.0003591

Legendre P, Birks HJB (2012) Chapter 8: From Classical to Canonical Ordination. In: Birks HJB, Lotter AF, Juggins S, Smol JP (eds) Track. Environ. Chang. Using Lake Sediments. Springer Netherlands, pp 201–248

Legendre P, Gallagher ED (2001) Ecologically meaningful transformations for ordination of species data. Oecologia 129:271–280. doi: 10.1007/s004420100716

Levy EM, Strain PM (1982) The composition of the preen gland waxes of some marine birds: A word of caution for chemotaxonomists. Comp Biochem Physiol Part B Comp Biochem 72:255–260. doi: http://dx.doi.org/10.1016/0305-0491(82)90043-8

Mardon J, Saunders SM, Anderson MJ, et al (2010) Species, gender, and identity: cracking petrels' sociochemical code. Chem Senses 35:309–21. doi: 10.1093/chemse/bjq021

Martin GR (2012) Through birds' eyes: insights into avian sensory ecology. J Ornithol 153:S23–S48. doi: 10.1007/s10336-011-0771-5

McCue MD, Amitai O, Khozin-Goldberg I, et al (2009) Effect of dietary fatty acid composition on fatty acid profiles of polar and neutral lipid tissue fractions in zebra finches, *Taeniopygia guttata*. Comp Biochem Physiol A Mol Integr Physiol 154:165–72. doi: 10.1016/j.cbpa.2009.06.002

Mielnik MB, Herstad O, Lea P, et al (2002) Sensory quality of marinated frozen stored chicken thighs as affected by dietary fish fat and vitamin E. Int J food Sci Technol 37:29–39.

Miller WL, Bose HS (2011) Early steps in steroidogenesis: intracellular cholesterol trafficking. J Lipid Res 52:2111–35. doi: 10.1194/jlr.R016675

Noble RE, Stjernholm RL, Mercier D, Lederer E (1963) Incorporation of Propionic Acid Into a Branched-chain Fatty Acid of the Preen Gland of the Goose. Nature 199:600–601.

Oksanen J, Blanchet FG, Kindt R, et al (2013) vegan: Community Ecology Package.

Osada K, Hanawa M, Tsunoda K, Izumi H (2011) Alteration of mouse urinary odor by ingestion of the xenobiotic monoterpene citronellal. Chem Senses 36:137–147. doi: 10.1093/chemse/bjq104

Overland M, Skrede a (2012) Fatty acid composition, oxidative stability and sensory quality of meat from broiler chicken fed autolysate from bacteria grown on natural gas. J Anim Physiol Anim Nutr (Berl) 96:739–46. doi: 10.1111/j.1439-0396.2011.01202.x

Pan PR, Dilworth BC, Day EJ, Chen TC (1979) Effect of season of the year, sex, and dietary fats on broiler performance, abdominal fat, and preen gland secretion. Poult Sci 58:1564–1574.

Piault R, Gasparini J, Bize P, et al (2008) Experimental support for the makeup hypothesis in nestling tawny owls (*Strix aluco*). Behav Ecol 19:703–709. doi: 10.1093/beheco/arm152

R Core Team (2014) R: A Language and Environment for Statistical Computing. Vienna, Austria

Reneerkens J, Piersma T, Damsté JSS (2002) Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why? Proc R Soc B Biol Sci 269:2135–2139. doi: 10.1098/rspb.2002.2132

Reneerkens J, Piersma T, Damsté JSS, et al (2007) Expression of Annual Cycles in Preen Wax Composition in Red Knots: Constraints on the Changing Phenotype. J Exp Zool 139:127–139.

doi: 10.1002/jez.a

Reneerkens J, Piersma T, Damsté JSS (2006) Discerning adaptive value of seasonal variation in preen waxes: comparative and experimental approaches. Acta Zool Sin 52:272–275.

Reneerkens J, Piersma T, Damsté JSS (2005) Switch to diester preen waxes may reduce avian nest predation by mammalian predators using olfactory cues. J Exp Biol 208:4199–4202. doi: 10.1242/jeb.01872

Rich T, Hurst J (1998) Scent marks as reliable signals of the competitive ability of mates. Anim Behav 56:727–735. doi: 10.1006/anbe.1998.0803

Sandilands V, Powell K, Keeling L, Savory CJ (2004) Preen gland function in layer fowls: factors affecting preen oil fatty acid composition. Br Poult Sci 45:109–115. doi: 10.1080/00071660410001668932

Schellinck HM, Slotnick BM, Brown RE (1997) Odors of individuality originating from the major histocompatibility complex are masked by diet cues in the urine of rats. Anim Learn Behav 25:193–199. doi: 10.3758/BF03199058

Shaw CL, Rutter JE, Austin AL, et al (2011) Volatile and Semivolatile Compounds in Gray Catbird Uropygial Secretions Vary with Age and Between Breeding and Wintering Grounds. J Chem Ecol 37:329–339. doi: 10.1007/s10886-011-9931-6

Shin D, Narciso-Gaytán C, Park JH, et al (2011) Dietary combination effects of conjugated linoleic acid and flaxseed or fish oil on the concentration of linoleic and arachidonic acid in poultry meat. Poult Sci 90:1340–7. doi: 10.3382/ps.2010-01167

Stevens L (1996) Avian Biochemistry and Molecular Biology. Cambridge University Press

Tabachnick BG, Fidell LS (2006) Using Multivariate Statistics, 5th Edition. Allyn & Bacon, Inc., Needham Heights, MA, USA

Tang BY, Hansen IA (1972) Lipogenesis in chicken uropygial glands. Eur J Biochem 31:372–7.

Thomas RH, Price ER, Seewagen CL, et al (2010) Use of TLC-FID and GC-MS／FID to examine the effects of migratory state, diet and captivity on preen wax composition in White-throated Sparrows *Zonotrichia albicollis*. Ibis (Lond 1859) 152:782–792.

Valenta JG, Rigby MK (1968) Discrimination of the odor of stressed rats. Science 161:599–601. doi: 10.1126/science.161.3841.599

Whittaker DJ, Soini HA, Gerlach NM, et al (2011) Role of Testosterone in Stimulating Seasonal Changes in a Potential Avian Chemosignal. J Chem Ecol 37:1349–1357. doi: 10.1007/s10886-011-0050-1

Yamazaki K, Boyse E a, Bard J, et al (2002) Presence of mouse mammary tumor virus specifically alters the body odor of mice. Proc Natl Acad Sci U S A 99:5612–5615. doi: 10.1073/pnas.082093099

Zhang Y-H, Du Y-F, Zhang J-X (2013) Uropygial gland volatiles facilitate species recognition between two sympatric sibling bird species. Behav Ecol 24(6):1271–1278. doi: 10.1093/beheco/art068

Chapter 4

Can preen wax odours signal genetic make-up in the South Island robin?

Abstract: *I tested for the presence of a heterozygosity signal, and the relationship between chemical distances and genetic distances, in the preen wax profiles of 69 male South Island robins originating from two severely bottlenecked populations that were experimentally outcrossed. Number of peaks, minimum retention time and retention time range did not differ significantly between "inbred" and "hybrid" individuals, while multivariate techniques (PERMANOVA and adonis) detected a significant effect of status (inbred vs. hybrid) in chord-transformed chemical profiles, but not on normalised profiles. No correlation was found between individual heterozygosity (measured as Internal Relatedness) and chemical profile, and no significant correlation was detected between chemical distances and genetic distances in 2211 dyads. However, chemical distances did separate birds that originated from the two populations. These results suggest that South Island robins preen wax could encode a geographic signal but only weak support for a heterozygosity signal.*

Introduction

At the broadest scale, chemical signals can function in the recognition of conspecifics and to assess the current breeding status (e.g. estrus) of a potential mate (Johnston 1979; Wyatt 2003; Bagley et al. 2006; Archunan & Rajagopal 2013). However, in some species one individual can potentially also assess another individual's quality and status via a number of chemical cues. For example, odours from males with higher testosterone concentrations are preferred by females in domestic goats (Longpre & Katz 2011), meadow voles (Ferkin et al. 1994), brown rats (Taylor et al. 1982), and hamsters (Johnston 1979). In male elephants, differing testosterone levels result in differences in the chemical composition of the temporal secretions, which in turn elicited different responses in conspecific males (Rasmussen et al. 2002). Similarly, alteration of vitamin D levels in males of the Iberian wall lizard caused changes in the chemical composition of their femural secretions and this elicited corresponding changes in the behaviour of females (Martín & López 2006). A higher proportion of oleic acid in the femoral secretions, thought to be positively correlated with body condition, also elicited stronger chemosensory responses from females in tongue-flicking bioassays (Martín & Lópezs 2010).

Apart from characters that reflect phenotypic traits (such as testosterone levels), chemical signals could be useful in assessing characters more directly linked to the bearer’s genotype. For example, there is evidence that mate choice can be driven by within-pair genetic compatibility rather than a genotype that signals universal mate quality (Tregenza & Wedell 2000; Mays & Hill 2004). Are chemical signals able to function in this type of preference as well? One such direct link between olfactory discrimination and the genetic make-up of an individual is mediated by genes of the Major Histocompatibility Complex (MHC), and has been found in rats (Brown et al. 1987; Schaefer et al. 2002), mice (Willse et al. 2006; Kwak et al. 2009), mandrills (Setchell et al. 2011), and humans (Eggert et al. 1999). In all these species, females prefer males with dissimilar MHC alleles and this choice is mediated by differences in odour profiles that reflect differences in MHC genes. A relationship between genetic make-up and non-MHC odour profiles has also been identified in the context of olfactory-mediated kin discrimination (Ferkin et al. 1997; Olsen et al. 1998; Heth et al. 2003; Boulet et al. 2009) and mate choice (Brown and Eklund 1994; Reusch et al., 2001, in Bonneaud et al. 2006; Parrott et al. 2007).

Notwithstanding the controversy over the nature of kin recognition (Grafen 1990; Barnard 1991), it is worthwhile to highlight the difference in the mechanisms reported as effecting kin recognition in non-avian vertebrates (i.e. primarily chemosignals) *vs.* birds (visual and auditory cues). Especially for mammals, and mice in particular, chemosignals appear to outweigh other

sensory modes in the literature investigating genetic relatedness (Barnard & Aldhous 1991; Eggert et al. 1999; Zavazava & Eggert 1997). By contrast, olfactory cues as effectors of kin discrimination or mate choice in birds have been neglected until recently (Komdeur & Hatchwell 1999; Nakagawa & Waas 2004). In the last 10 years, renewed interest on olfaction in birds (Bonadonna & Mardon 2013) has led to a number of studies investigating avian chemosignals and their relationship to individual genetic make-up (Coffin et al. 2011; Célérier et al. 2011; Krause et al. 2012; Leclaire et al. 2012; Bonadonna & Sanz-Aguilar 2012; Leclaire et al. 2014). The growing recognition of the importance of olfactory signals in birds suggests that such cues may function in a variety of social situations and play a key role in how birds might assess the genetic relatedness, levels of genetic diversity and the degree of genetic compatibility of conspecifics.

In this context, I explored the relationship between preen wax profile and genetic make-up in an isolated population of South Island Robins, *Petroica australis*. Robins appear to recognise and respond specifically to the odour of conspecific preen wax, suggesting that at least some components of this secretion may also function as chemical signals (Thierry 2014). The population of robins I used for my study provided a unique opportunity to assess how preen wax composition (and thus odour) is related to levels of genetic diversity at an individual level. This is because the population experienced a severe bottleneck in the past, but has recently been the subject of a genetic rescue, i.e. translocation of individuals from a neighbouring island (Heber et al. 2013). Thus, I could compare the preen wax and odour profiles from individuals having different levels of genetic diversity as well as geographic origin, to test if preen wax encodes a heterozygosity signal. I also tested whether genetic distance, calculated based on 36 sequenced microsatellite loci, correlated with chemical distance in 2211 male-male dyads, to determine if an individual's genetic profile correlated with its odour profile, and thus whether preen wax odours could function as a chemical signal of relatedness or genetic similarity.

Methods

Study species and population

The South Island robin is a medium sized, insectivorous, ground-feeding passerine endemic to New Zealand (Heather & Robertson 2000). Robins are sedentary and are reluctant to cross even a few hundred meters of open ground or water (Flack 1979). Once common across the South Island, its numbers have declined since European settlement mainly due to deforestation and introduction of mammalian predators (Miller & Lambert 2006). The South Island robin has been used to trial reintroduction experiments to offshore islands, beginning in the 1970s (Armstrong 2000). The

population used in this study is the result of one such reintroduction attempts. Motuara Island is a small (~ 80 hectares) island located in the Marlborough Sounds, New Zealand. Its forest cover was burnt off at the start of the 20th century and then the island was used to graze sheep and as an experimental goat farming station. This activity was discontinued around 80 years ago, and the island went through a phase of natural reforestation and management to remove exotic predators. In 1973, 5 robins were transferred from nearby Nukuwaiata Island (Boessenkool et al. 2007), and a stable population established from this single founding event. As robins are reluctant to cross open spaces, this population was isolated from external immigration. The population increased to approximately 300 adults by 2008 (Heber 2012), but due to the extreme bottleneck of the founding event, the population showed signs of inbreeding depression (Hale & Briskie 2007).

To alleviate the effects of inbreeding depression, an experimental translocation using females from Allports Island—a nearby, bottlenecked island population of robins that was also founded in the 1970s by 5 birds from a source population near Kaikoura—was carried out in 2008, to test the efficiency of genetic rescue using an inbred population as donor (Heber et al. 2013). This experiment gave rise on Motuara Island to what is essentially a "mixed" population, composed of a handful of translocated females from Allports Island, a large number of original, "inbred" Motuara birds, and some inter-population "hybrids". Each bird I sampled was therefore assigned a "status" category of either "inbred" (the original Motuara Island birds) or "hybrid" (the offspring of crosses between Motuara robins and Allports robins). Recaptured birds were classified based on pedigree information obtained during the original translocation experiment (Heber et al. 2013) while newly captured individuals were classified based on their allelic composition, via DAPC (see section "genetic analysis"). Note that purebred Motuara birds, descending from the 5 individuals robins that founded the population in 1973, are termed "inbred" in this study, even though they might not be the product of recent within-family mating. The category "hybrid" comprises: a) birds that are the product of a mating between an "inbred" Motuara bird and an "inbred" Allports bird, defined as F1 hybrids, b) birds that are the product of a mating between F1 hybrids, defined as F2 hybrids, and c) birds classified as "hybrids" by DAPC. The majority of birds found on the island were "inbred", owing to the numerical nature and recent history of the translocation. I targeted known hybrids during my sampling, and was able to recapture 6 male hybrids (four F1 and one F2 hybrids). Out of the 43 newly captured (i.e. unknown) individuals, DAPC classified 2 as hybrids and the remaining as "inbred". Heterozygosity levels (as measured by standardized heterozygosity) differed significantly (*t*-test: $t = 2.741$, df = 65, p = 0.003) between the two groups, with hybrid robins having higher SH (mean ± sd: 1.24 ± 0.18) than the inbred robins (mean ± sd: 0.99 ± 0.25).

Sample collection

From 24 August to 1 September 2012, 69 male South Island Robins were captured on Motuara Island using clap traps. All birds were adults, and as robins breed at one year of age, all were sexually mature when sampled. I only sampled males to avoid the confounding effects of sexual differences in preen wax composition. All newly captured individuals (43/69) were fitted with a metal band and a combination of 3 colour bands for individual identification. A total of 26 were recaptured individuals, which had been banded and genotyped during a previous study (Heber 2012). The sampling period fell within the early breeding season for the species at the sampling location. Each bird was handled using a new pair of latex gloves and placed in a clean paper bag before processing. This precaution was taken to minimise contamination of the preen wax samples with oil from the hands of the researchers. Preen wax samples were collected from each bird by gently squeezing the uropygial gland with wax-coated forceps tips; the extruded droplet was then collected on a clean stainless-steel inoculation loop and placed in a clean glass insert lodged inside a glass vial, sealed with teflon-covered lids to minimise evaporation or contamination of samples. Vials were kept cool using freezer packs in the field, until later frozen. Samples were kept at -20°C until analysis. Blood samples were collected for the newly captured birds via brachial venipuncture, and approximately 10–30 µl of blood was stored in 1 ml of Queen's Lysis Buffer (0.01 M Tris-Hcl, 0.01 M NaCl, 0.01 M Na-EDTA(pH 7.5), 1% (v/v) n-Lauroylsarcosine; pH7.5; Seutin et al.1991) at room temperature.

Sample preparation, chemical analysis and post-processing of chromatographic data

Samples were dissolved in 100 µl of ethyl acetate, poured directly into the insert containing the inoculation loop and the preen wax. The vial was then re-capped, and vortexed for 60 seconds at 700 rpm to ensure dissolution of the preen wax. The original cap was then substituted with a chromatographic cap fitted with a single-use PTFE silicone septum. Samples were analysed on a Shimadzu GC-2010 gas chromatograph, equipped with a Shimadzu AOC-20i+s auto-injector and a Varian CP-SIL 5 CB capillary column (25 m length x 320 µm internal diameter x 0.12 µm film thickness). Injection volume was 1 µl, with a 6:1 split ratio. Injection port temperature was set at 250°C, the carrier gas was nitrogen with a total flow of 19.0 ml/min and a linear velocity of 36.7 cm/sec. The FID detector operated at 320°C, with a sampling interval of 40 msec. Oven temperature was programmed as follows: initial temperature 70°C with a hold time of 4 mins, then increased to 130°C at a rate of 20°C/min, and finally increased to 320°C at 4°C/min rate (hold time 15 minutes). Results were recorded on Shimadzu's GCSolution, version 2.3 (©Shimadzu 2002-2009) software.

As it was not feasible to standardise the quantity of preen wax extracted from each bird, I

used the relative proportions of compounds rather than their absolute quantities. Only peaks whose area comprised on average at least 0.1% of the profile were retained for statistical analysis. These compounds constituted, on average, 99.8 ± 0.3 % of the absolute chromatogram area; they were present in every individual – as assessed by visual inspection of every chromatogram – but not all of them were present in every chromatogram above the detection limit of the instrument. Peaks that were present on a chromatogram but fell below the detection limit of the instrument were given an arbitrary value of 0.000001.

Genetic analysis

Blood samples were genotyped at the Max Planck Institute for Ornithology, Germany. Thirty-two autosomal microsatellites loci were sequenced, according to the procedure detailed in Appendix A, Heber (2012). Samples belonging to newly captured birds were genotyped in 2013, while samples belonging to recaptured birds had been genotyped, at the same facility and applying the same technique, in 2011. The resulting information was used to calculate measures of heterozygosity within the R environment (R Core Team 2014). The function mlh in package Rhh (Alho & Välimäki 2012) was used to obtain three measures of multilocus heterozygosity: (1) homozygosity by loci (HL), (2) internal relatedness (IR), and (3) standardized heterozygosity (SH). These allowed me to classify each individual along a continuous gradient of heterozygosity. The three measures of heterozygosity derived by function mlh were highly correlated (Table 4.1). Therefore, I used only Internal Relatedness (IR) for all subsequent analyses. Additionally, microsatellite allelic composition was used to infer the origin of newly captured bird, to be able to classify unknown individuals as “inbred” or “hybrid” using Discriminant Analysis of Principal Coordinates (Jombart et al. 2010), implemented in the R package adegenet (Jombart 2008).

Table 4.1: Spearman's correlation coefficients, confidence intervals and p values for the correlations among internal relatedness (IR), standardised heterozygosity (SH) and homozygosity by loci (HL).

	Lower C.I	**Correlation coefficient**	**Upper C.I.**	**p**
IR-SH	-0.98	-0.97	-0.95	< 0.001
IR-HL	0.94	0.97	0.98	< 0.001
SH-HL	-0.99	-0.98	-0.97	< 0.001

Statistical analysis

The influence of “status” (inbred *vs.* hybrid) and of heterozygosity (expressed as IR) on the

chemical profiles of birds were analysed using two multivariate techniques: (1) permutational MANOVA ("non parametric MANOVA", after Anderson (2001), implemented in R, package vegan (Oksanen et al. 2013b, function adonis) and (2) Canonical Analysis of Principal Coordinates (CAP, Anderson & Willis 2003), implemented in R, package vegan (Oksanen et al. 2013a), function capscale). These two techniques share some similarities, but each is expected to perform slightly better in different situations, depending on the data structure and the correlation matrix of the dependent variables (see Chapter 3). As discussed in Anderson & Robinson (2003) and Anderson (2006), the choice of transformation and dissimilarity measure used in multivariate analysis strongly influences the results, as different metrics emphasise different aspects of the chemical species abundance matrix. I therefore followed the method of Leclaire et al. (2014), and carried out my analyses using both (1) Euclidean distances calculated on standardised relative proportions, which give equal importance to all compounds present in the profile, and (2) chord distances, which emphasise the contribution of the compounds which show large absolute differences amongst individuals, and are in general the most abundant compounds. This allowed me to focus on two different aspects of the same dataset and discern which peaks were primarily responsible for the differences in multivariate composition. I further followed the method of Leclaire et al. (2012, 2014) and analysed (1) the relationship between the PC scores (from a Principal Component Analysis carried out on standardised relative abundances of compounds) and a measure of genetic diversity (IR), and (2) the relationship between a set of chemical distances (chord distances and Euclidean distances on normalised profiles) and genetic distances (Queller-Goodnight distances) for male-male dyads via a Mantel test. All analyses were carried out in R, version 3.1.2 (R Core Team 2014).

Results

Differences between "inbred" and "hybrid" robins

For each sample, I calculated the number of peaks whose relative area was higher than 0.1% and performed a *t*-test to investigate differences between "hybrid" and "inbred" birds. The mean peak richness (± standard deviation) was 13.38 ± 4.72 for hybrid robins and 15.78 ± 6.28 for inbred robins and was not significantly different (*t*-test: $t = -1.0413$, df = 65, p = 0.30). Homoscedasticity was checked via Levene's test ($F_{1,65} = 2.02$, p= 0.16).

I then calculated the mean retention time as the sum of the weighted retention time (RT * relative peak area) of each peak present in the profile. The mean retention time (± standard deviation) was 24.20 ± 0.35 minutes for hybrid robins and 24.38 ± 0.37 minutes for inbred robins.

The difference was not significant (*t*-test: t = -1.271, df = 65, p = 0.21). Homoscedasticity was checked via Levene's test ($F_{1,65}$ = 0.08, p = 0.77).

The range of retention times (mean ± standard deviation) was 36.95 ± 5.11 minutes for hybrids and 39.12 ± 5.86 minutes for inbred birds and did not differ significantly between the two groups (*t*-test: t = -0.997, df = 65, p = 0.32). Homoscedasticity was checked via Levene's test ($F_{1,65}$ = 0.13, p = 0.72).

PERMANOVA and CAP run on chord distances detected a significant difference between the profiles of hybrid and original birds (adonis, status: pseudo-$F_{1,66}$ = 5.562, R^2 = 0.08, P = 0.0095, permutations = 9999; capscale, status: pseudo-$F_{1,66}$ = 5.562, P = 0.0096, permutations = 9999, Figure 4.1a). Table 4.2 shows the coefficients extracted from adonis for the factor “status” and the peaks scores extracted from capscale: these indicate the contribution to the model for each variable (in case of adonis) and to the discriminant axis CAP1 (for capscale).

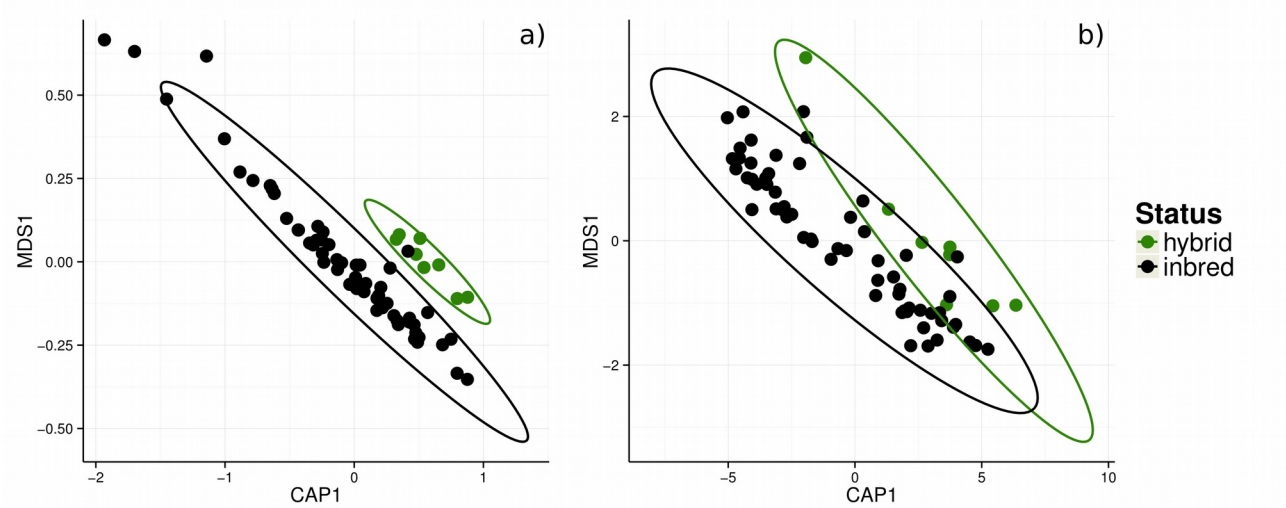

Figure 4.1: CAP plot for a) chord-transformed peak area and b) normalized peak area of “original” and “hybrid” Motuara robins.

The analyses conducted on normalised relative proportions were not significant (adonis, status: pseudo-$F_{1,66}$ = 1.329, R^2 = 0.02, P = 0.199, permutations = 9999; CAP, status: pseudo-$F_{1,66}$ = 1.329, P= 0.197, permutations = 9999, Figure 4.1b).

As the above techniques are sensitive to both location and dispersion effects, I checked for multivariate homogeneity of group dispersion via function betadisper (a multivariate analogue of Levene's test), to confirm that the significant result returned by the analysis on chord distances was not caused by multivariate heteroscedasticity. Group dispersions were not significantly different (betadisper, status: pseudo-$F_{1,66}$ = 2.920, P= 0.092), but given the difference in sample sizes for the

two groups, I re-ran the analysis using random resampling of the largest group to perform 200 capscale tests with $n_{hybrids} = n_{originals} = 8$, and plotted the distribution of the resulting p-values (Figure 4.2). The median p-value obtained with this resampling scheme was 0.007, confirming the result of the previous analysis. Moreover, all replicates returned the highest score for peak 24, confirming its importance in separating the two groups of robins (Figure 4.3).

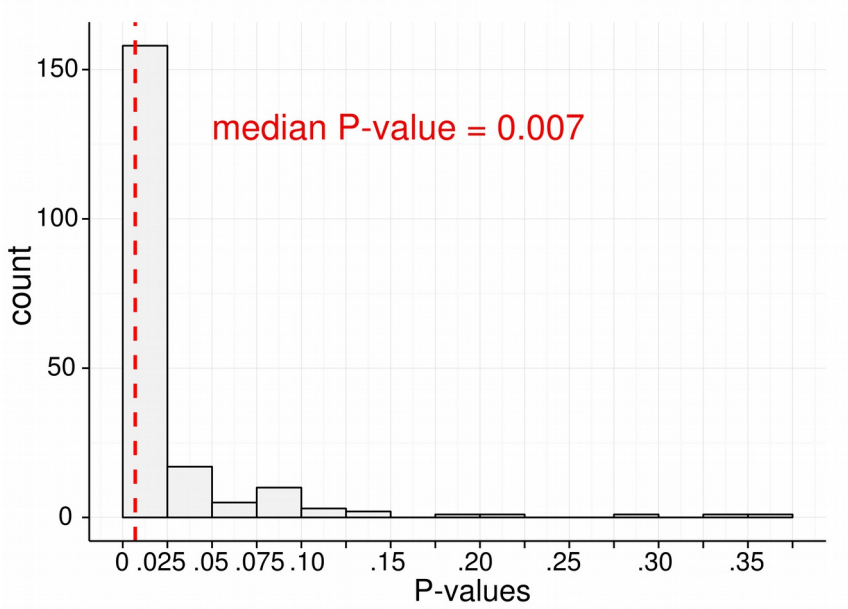

Figure 4.2: Distribution of the CAP p-values obtained from random resampling of the "original" subgroup, where 200 subsamples of size = 8 were drawn.

Table 4.2: Adonis model coefficients and loadings on the discriminant capscale axis for the chromatographic peaks included in the 2 analyses (on chord transformed relative peak areas). The coefficients and the loadings are presented in decreasing order.

Peak number	**Adonis coefficients**	**CAP1 loadings**
24	-0.018	-0.968
15	0.003	0.183
2	0.002	0.092
26	-0.002	-0.081
36	0.001	0.064
40	0.001	0.046
32	-0.001	-0.040
21	-0.001	-0.036
20	-0.001	-0.036
12	0.000	-0.021
34	0.000	-0.020

67	0.000	-0.019
50	0.000	0.018
31	0.000	-0.017
5	0.000	0.017
6	0.000	0.015
3	0.000	0.012
33	0.000	-0.012
69	0.000	-0.011
9	0.000	0.008
28	0.000	-0.007
19	0.000	-0.007
8	0.000	-0.006
38	0.000	-0.005
10	0.000	-0.005
51	0.000	0.004
7	0.000	-0.003
39	0.000	-0.001
53	0.000	0.001
11	0.000	-0.001
72	0.000	-0.001
66	0.000	0.001
71	0.000	-0.001
13	0.000	-0.001
48	0.000	-0.001
47	0.000	-0.001
16	0.000	0.000

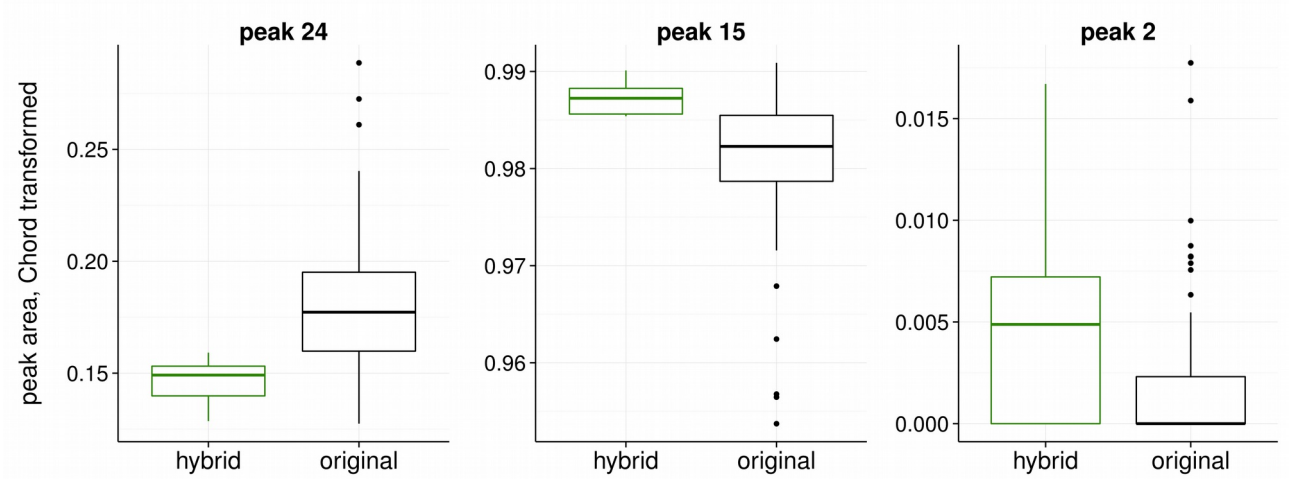

Figure 4.3: Boxplots of the 3 most discriminant peaks according to adonis model and CAP analysis

Influence of heterozygosity (IR) on chemical profiles

PERMANOVA and CAP on chord distances did not detect a significant correlation between the IR scores and the chemical profiles of individual birds (adonis, IR: pseudo-$F_{1,64}$ = 1.340, R^2 = 0.02, P = 0.243, permutations = 9999; CAP, status: pseudo-$F_{1,64}$ = 1.341, P = 0.240, permutations = 9999). The analyses on the normalised relative proportions were also not significant (adonis, IR: pseudo-$F_{1,64}$ = 0.519, R^2 = 0.008, P = 0.873, permutations = 9999; CAP, status: pseudo-$F_{1,64}$ = 0.519, P = 0.872, permutations = 9999). The absence of a clear correlation between CAP scores and IR values can be appreciated in Figure 4a (chord-transformed data) and 4b (normalised data).

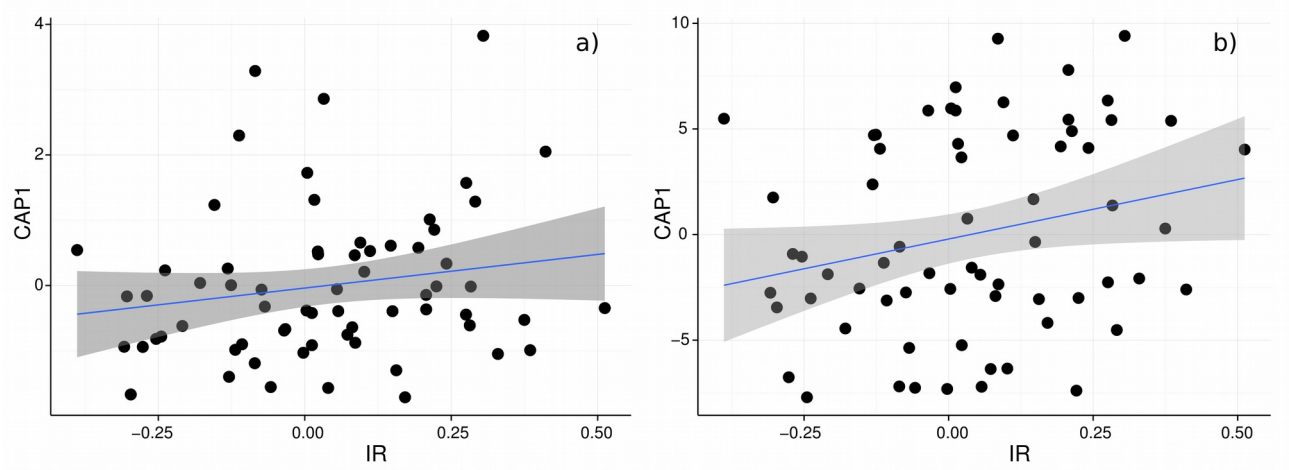

Figure 4.4: Relationship between genetic heterozygosity (IR) and CAP scores for a CAP analysis run on a) chord-transformed data and b) normalized data. Blue lines are linear least squares regression fits to data points, and the gray shading shows 95% confidence intervals for the regression line.

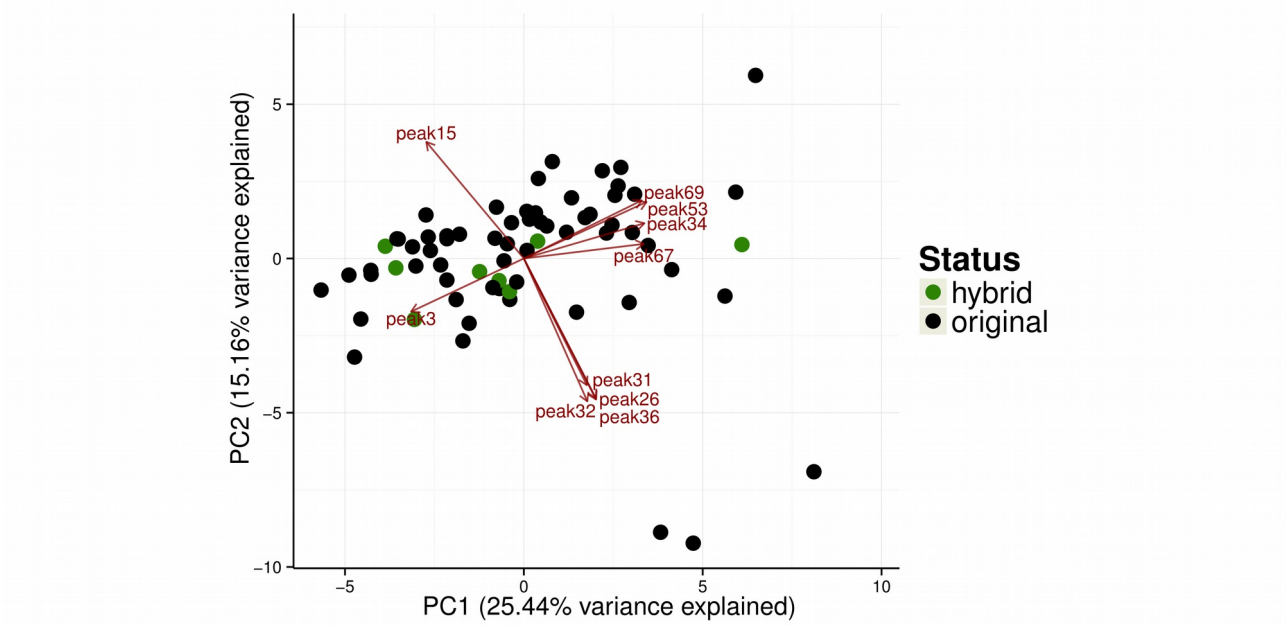

FIgure 4.5: Bipot of PCA on relative peak areas. Arrows indicate the contribution of the peaks with the 5 highest loadings on PC1 and PC2.

Figure 4.5 shows the biplot of the first two axes extracted from a Principal Component Analysis on relative peak areas (function prcomp in base R, variables centered and scaled). The correlation of each gas-chromatographic peak with PC1 and PC2 is presented in Table 4.3. As can be seen from Figure 4.5 and Table 4.3, the first axis correlates positively with some of the last eluting compounds (high loadings of PC1 on peak 53, peak 67 and 69) and also has a strong negative correlation with peak 3, one of the earliest eluting compounds. PC2 is largely influenced (in an inverse fashion) by mid-eluting peaks, due to high negative loadings on peaks 26, 32 and 36.

Table 4.3: Chromatographic peaks used in the PCA and their correlation with PC1 and PC2

Peak number	**PC1**	**PC2**
2	-0.108	-0.132
3	-0.221	-0.121
5	0.164	-0.012
6	0.207	0.007
7	0.116	-0.035
8	0.093	0.141
9	0.007	0.195
10	0.209	0.162
11	0.035	0.065

12	0.147	0.184
13	0.085	0.130
15	-0.191	0.264
16	-0.219	-0.118
19	0.204	0.167
20	0.197	0.071
21	-0.114	-0.032
24	0.063	-0.010
26	0.141	-0.317
28	0.183	0.067
31	0.123	-0.287
32	0.124	-0.324
33	0.207	-0.204
34	0.236	0.080
36	0.141	-0.320
38	0.110	0.065
39	0.115	0.125
40	0.182	-0.263
47	0.173	0.095
48	0.085	0.130
50	0.211	-0.223
51	0.147	0.109
53	0.240	0.128
66	0.198	0.092
67	0.235	0.033
69	0.231	0.132
71	0.106	-0.152
72	0.106	-0.152

PC1 and PC2 scores for each individual were extracted from the PCA and tested for correlation with the individual's IR: there was no significant correlation between individual levels of heterozygosity, as measured by IR, and either PC1 or PC2 scores (PC1: $F_{1,64} = 0.157$, P = 0.693; PC2: $F_{1,64} = 0.3$, P = 0.586).

Matrix correlation of chemical and genetic distances

Chemical distances calculated on normalised relative proportions were not significantly correlated with genetic distances in 2211 male-male dyads (Mantel test: r = -0.0272, P = 0.710, n = 2211 dyads, Figure 4.6a). The analysis conducted on chord distances was also not significant (Mantel test: r = -0.0155, P = 0.624, n = 2145 dyads, Figure 4.6b).

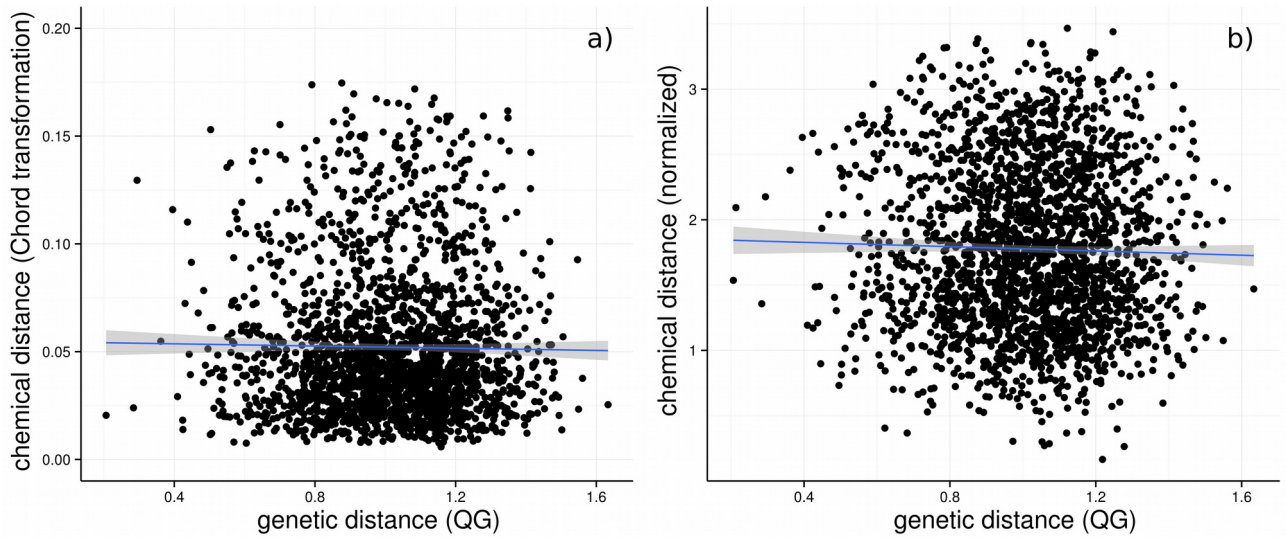

Figure 4.6: Relationship between genetic distance (Queller-Goodnight) and chemical distance between male-male dyads calculated on a) chord-transformed and b) normalized data. Blue lines are linear least squares regression fits to data points, and the gray shading shows 95% confidence intervals for the regression line.

Discussion

The combination of genetic and chemical data allowed me to show that it is possible to distinguish, based on their chemical profile, inbred South Island robins on Motuara Island from sympatric hybrid individuals that are the product of crossing between birds from the same lineage and birds from a different, genetically distinct population. This difference was significant in spite of the small number of hybrids included in the analysis, and was confirmed on a sub-analysis with equal samples sizes obtained through random resampling. Interestingly, this difference does not seem to be linked to differences in heterozygosity levels, as the correlation between the chemical profiles of birds and their Internal Relatedness was not significant. The peaks driving the difference between the two groups could therefore reflect the different genetic structure of each group, rather than carrying information about an individual's level of heterozygosity. It is possible that, in robins, the chemical profile encodes a “geographic signal” - a trace indicative of the geographical provenance of the individuals sampled - rather than a “heterozygosity signal”. Chemical profiles have been

shown to diverge in geographically separated populations of social insects (Martin et al. 2011; Bonelli et al. 2015), and non-chemical avian signals also show geographic variation (Searcy et al. 2015).

Carrying out this analysis on Motuara Island allowed me access to a population that had undergone experimental outcrossing, where I could sample birds whose lineage and levels of genetic variation were different, but which live in the same environment. Previous studies (Leclaire et al. 2012; Leclaire et al. 2014) have only been able to test the relationship between measures of heterozygosity and chemical profile, only exploring one of the possible aspects of the topic. This relationship was not significant in my sample, and there are several possible explanations for this. Unlike what is found in kittiwake *Rissa tridactyla* (Leclaire et al. 2012), it is possible that chemical profiles are not used a signal to code for heterozygosity in South Island Robins. If robins do assess a conspecific's genetic diversity/ genetic quality, they may do so via visual and/or acoustic means.

Secondly, it is necessary to point out that heterozygosity levels in this study were measured using microsatellites: the indexes I calculated convey information about neutral genetic diversity, and this may not always directly related to functional genetic diversity (Holderegger et al. 2006). Furthermore, it is presently unknown whether neutral genetic diversity or functional genetic diversity measured at other loci is related to diversity in preen wax profiles in birds. Mounting evidence indicates that such chemical profiles have a strong genetic basis (Bonadonna et al. 2007; Mardon et al. 2010; Leclaire et al. 2012) but the genes coding for preen wax profiles have not been isolated, therefore even generic measures of functional diversity could still fail to carry information that relates to diversity in chemical profiles.

Finally, it must be remembered that the Motuara Island population of robins was founded by 5 individuals only. It is possible that such a severe bottleneck has reduced both the chemical diversity and the range of genetic diversity that the birds express (Ortego et al. 2008). The range of IR values is similar to what found by Leclaire (2012), but it is possible that chemical diversity was lost and that the birds chemical profile has lost some complexity, and therefore a relationship between heterozygosity and profile diversity, detected in a range of species (Charpentier et al. 2008; Ilmonen et al. 2009; Charpentier et al. 2010; Leclaire et al. 2012), was not found in the Motuara robins.

Studies sampling robins from a number of populations across New Zealand have shown remarkable diversity in the chemical profiles (Kempenaers, *pers. comm.*). These populations are reproductively isolated: the differences could reflect local adaptations, and in this context the ability to recognize a foreign bird would carry a selective advantage, in terms of avoiding outbreeding depression. The reported geographic variation is unlikely to be due to geographic differences in diet

or climate, as the birds I sampled were living on the same island.

These findings call for more research into robins' olfactory discrimination, and the degree to which they are able to perceive and make use of differences in chemical profiles. It would be interesting to test whether robins are able to use olfactory information to differentiate between sympatric and allopatric individuals. Robins have already been shown to recognise and respond to the odour of conspecific preen wax (Thierry 2014), and other avian species are able to discriminate individuals of different sexes and subspecies based on odour profiles (Mihailova et al. 2014): it seems therefore plausible that the information I found at the chemical levels can be effectively transmitted at the physiological level and used by robins to modulate their social behaviour.

References

Alho, J. & Välimäki, K., 2012. Rhh: Calculating multilocus heterozygosity and heterozygosity-heterozygosity correlation. Available at: http://cran.r-project.org/package=Rhh.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, 26, pp.32–46.

Anderson, M.J., 2006. Distance-based tests for homogeneity of multivariate dispersions. *Biometrics*, 62(1), pp.245–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16542252 [Accessed July 15, 2014].

Anderson, M.J. & Robinson, J., 2003. Generalized Discriminant Analysis Based on Distances. *Australian & New Zealand Journal of Statistics*, 45(3), pp.301–318.

Anderson, M.J. & Willis, T.J., 2003. Canonical Analysis of Principal Coordinates: a Useful Method of Constrained Ordination for Ecology. *Ecology*, 84(2), pp.511–525.

Archunan, G. & Rajagopal, T., 2013. Detection of estrus in Indian blackbuck: Behavioural, hormonal and urinary volatiles evaluation. *General and comparative endocrinology*, 181, pp.156–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23229002.

Armstrong, D.P., 2000. Reintroductions of New Zealand robins: a key component of ecological restoration. *Reintroduction News*, 19, pp.44–47.

Bagley, K.R. et al., 2006. Male African elephants, *Loxodonta africana*, can distinguish oestrous status via urinary signals. *Animal Behaviour*, 71(6), pp.1439–1445. Available at: http://www.sciencedirect.com/science/article/pii/S0003347206001321.

Barnard, C., 1991. Kinship and social behaviour: The trouble with relatives. *Trends in ecology & evolution (Personal edition)*, 6(October), pp.310–312.

Barnard, C.J. & Aldhous, P., 1991. Kinship, kin discrimination and mate choice. In P. G. Hepper, ed. *Kin recognition*. Cambridge University Press, Cambridge, pp. 125–147.

Boessenkool, S. et al., 2007. Large mainland populations of South Island robins retain greater genetic diversity than offshore island refuges. *Conservation Genetics*, 8, pp.705–714.

Bonadonna, F. et al., 2007. Individual odor recognition in birds: An endogenous olfactory signature on petrels' feathers? *Journal of Chemical Ecology*, 33, pp.1819–1829.

Bonadonna, F. & Mardon, J., 2013. Besides Colours and Songs, Odour is the New Black of Avian Communication. In M. L. East & M. Dehnhard, eds. *Chemical Signals in Vertebrates 12*.

Springer New York, pp. 325–339. Available at: http://dx.doi.org/10.1007/978-1-4614-5927-9_26.

Bonadonna, F. & Sanz-Aguilar, A., 2012. Kin recognition and inbreeding avoidance in wild birds: the first evidence for individual kin-related odour recognition. *Animal Behaviour*, 84(3), pp.509–513. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0003347212002758 [Accessed January 21, 2014].

Bonelli, M. et al., 2015. Population diversity in cuticular hydrocarbons and mtDNA in a mountain social wasp. *Journal of chemical ecology*, 41(1), pp.22–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25516225 [Accessed August 2, 2015].

Bonneaud, C. et al., 2006. Complex MHC-based mate choice in a wild passerine. *Proceedings of the Royal Society B: Biological Sciences*, 273, pp.1111–1116.

Boulet, M., Charpentier, M.J.E. & Drea, C.M., 2009. Decoding an olfactory mechanism of kin recognition and inbreeding avoidance in a primate. *BMC evolutionary biology*, 9, p.281.

Brown, J.L. & Eklund, A., 1994. Kin Recognition and the Major Histocompatibility Complex: An Integrative Review. *The American Naturalist*, 143(3), p.435.

Brown, R.E., Singh, P.B. & Roser, B., 1987. The major histocompatibility complex and the chemosensory recognition of individuality in rats. *Physiology & behavior*, 40, pp.65–73.

Célérier, A. et al., 2011. Chemical kin label in seabirds. *Biology letters*, 7(6), pp.807–10. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3210654&tool=pmcentrez&rendertype=abstract [Accessed March 5, 2015].

Charpentier, M.J.E. et al., 2010. Message “scent”: lemurs detect the genetic relatedness and quality of conspecifics via olfactory cues. *Animal Behaviour*, 80(1), pp.101–108. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0003347210001351 [Accessed September 26, 2014].

Charpentier, M.J.E., Boulet, M. & Drea, C.M., 2008. Smelling right: The scent of male lemurs advertises genetic quality and relatedness. *Molecular Ecology*, 17(14), pp.3225–3233.

Coffin, H.R., Watters, J. V. & Mateo, J.M., 2011. Odor-based recognition of familiar and related conspecifics: A first test conducted on captive Humboldt penguins (*Spheniscus humboldti*). *PLoS ONE*, 6(9), pp.10–13.

Eggert, F., Müller-Ruchholtz, W. & Ferstl, R., 1999. Olfactory cues associated with the major histocompatibility complex. *Genetica*, 104, pp.191–7.

Ferkin, M.H. et al., 1994. Attractiveness of male odors to females varies directly with plasma testosterone concentration in meadow voles. *Physiology and Behavior*, 55(2), pp.347–353.

Ferkin, M.H. et al., 1997. Attractiveness of scents varies with protein content of the diet in meadow voles. *Animal Behaviour*, 53(1), pp.133–141. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0003347296902845.

Flack, J.A.D., 1979. Biology and ecology of the South Island robin. In D. Hunt & B. J. Gill, eds. *Ecology of Kowhai Bush, Kaikoura*. University of Canterbury, pp. 22–26.

Grafen, A., 1990. Do animals really recognize kin? *Animal Behaviour*, 39, pp.42–54.

Hale, K. a. & Briskie, J. V., 2007. Decreased immunocompetence in a severely bottlenecked population of an endemic New Zealand bird. *Animal Conservation*, 10(1), pp.2–10. Available at: http://doi.wiley.com/10.1111/j.1469-1795.2006.00059.x [Accessed April 14, 2015].

Heather, B.D. & Robertson, H.A., 2000. *The field guide to the brids of New Zealand*, Auckland,

New Zealand: Viking.

Heber, S. et al., 2013. The genetic rescue of two bottlenecked South Island robin populations using translocations of inbred donors. *Proceedings of the Royal Society B: Biological Sciences*, 280(1752), p.20122228. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23235701.

Heber, S., 2012. *Translocations and the "genetic rescue" of bottlenecked populations*. University of Canterbury, Christchurch, New Zealand.

Heth, G. et al., 2003. Genetic relatedness assessment through individual odour similarities in mice. *Biological Journal of the Linnean Society*, 78(4), pp.595–603. Available at: http://doi.wiley.com/10.1046/j.0024-4066.2002.00194.x.

Holderegger, R., Kamm, U. & Gugerli, F., 2006. Adaptive vs. neutral genetic diversity: implications for landscape genetics. *Landscape Ecology*, 21(6), pp.797–807. Available at: http://link.springer.com/10.1007/s10980-005-5245-9 [Accessed July 13, 2015].

Ilmonen, P. et al., 2009. Females prefer the scent of outbred males: good-genes-as-heterozygosity? *BMC evolutionary biology*, 9, p.104. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2698884&tool=pmcentrez&rendertype=abstract [Accessed January 21, 2014].

Johnston, R.E., 1979. Olfactory preferences, scent marking, and "proceptivity" in female hamsters. *Hormones and Behavior*, 13(1), pp.21–39. Available at: http://linkinghub.elsevier.com/retrieve/pii/0018506X79900321.

Jombart, T., 2008. adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, 24, pp.1403–1405.

Jombart, T., Devillard, S. & Balloux, F., 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC genetics*, 11(1), p.94. Available at: http://www.biomedcentral.com/1471-2156/11/94.

Komdeur, J. & Hatchwell, B.J., 1999. Kin recognition: Function and mechanism in avian societies. *Trends in Ecology and Evolution*, 14, pp.237–241.

Krause, E.T. et al., 2012. Olfactory kin recognition in a songbird. *Biology letters*, 8(3), pp.327–329. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3367747&tool=pmcentrez&rendertype=abstract [Accessed January 21, 2014].

Kwak, J. et al., 2009. Major histocompatibility complex-regulated odortypes: Peptide-free urinary volatile signals. *Physiology and Behavior*, 96(1), pp.184–188. Available at: http://dx.doi.org/10.1016/j.physbeh.2008.10.003.

Leclaire, S. et al., 2014. Preen secretions encode information on MHC similarity in certain sex-dyads in a monogamous seabird. *Scientific Reports*, 4, p.6920.

Leclaire, S. et al., 2012. Semiochemical compounds of preen secretion reflect genetic make-up in a seabird species. *Proceedings of the Royal Society B: Biological Sciences*, 279(1731), pp.1185–1193. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3267147&tool=pmcentrez&rendertype=abstract [Accessed January 21, 2014].

Longpre, K.M. & Katz, L.S., 2011. Estrous female goats use testosterone-dependent cues to assess mates. *Hormones and Behavior*, 59(1), pp.98–104. Available at: http://dx.doi.org/10.1016/j.yhbeh.2010.10.014 [Accessed April 14, 2015].

Mardon, J. et al., 2010. Species, gender, and identity: cracking petrels' sociochemical code. *Chemical senses*, 35(4), pp.309–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20190009 [Accessed January 21, 2014].

Martín, J. & López, P., 2006. Vitamin D supplementation increases the attractiveness of males' scent for female Iberian rock lizards. *Proceedings of the Royal Society B: Biological Sciences*, 273(July), pp.2619–2624.

Martín, J. & Lópezs, P., 2010. Condition-dependent pheromone signaling by male rock lizards: More oily scents are more attractive. *Chemical Senses*, 35, pp.253–262.

Martin, S.J., Helanterä, H. & Drijfhout, F.P., 2011. Is parasite pressure a driver of chemical cue diversity in ants? *Proceedings of the Royal Society B: Biological Sciences*, 278(1705), pp.496–503. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3025671&tool=pmcentrez&rendertype=abstract [Accessed August 13, 2015].

Mays, H.L. & Hill, G.E., 2004. Choosing mates : good genes versus genes that are a good fit. *Trends in Ecology & Evolution*, 19(10).

Mihailova, M. et al., 2014. Odour-based discrimination of subspecies, species and sexes in an avian species complex, the crimson rosella. *Animal Behaviour*, 95, pp.155–164. Available at: http://www.sciencedirect.com/science/article/pii/S0003347214002887.

Miller, H.C. & Lambert, D.M., 2006. A molecular phylogeny of New Zealand's Petroica (Aves: Petroicidae) species based on mitochondrial DNA sequences. *Molecular phylogenetics and evolution*, 40(3), pp.844–55. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16750641 [Accessed April 14, 2015].

Nakagawa, S. & Waas, J.R., 2004. O sibling, where art thou? a review of avian sibling recognition with respect to the mammalian literature. *Biological Reviews*, 79(1), pp.101–119. Available at: http://doi.wiley.com/10.1017/S1464793103006249 [Accessed March 5, 2015].

Oksanen, J. et al., 2013a. Package "vegan" *R package ver. 2.0–8*, p.264.

Oksanen, J. et al., 2013b. vegan: Community Ecology Package. Available at: http://cran.r-project.org/package=vegan.

Olsen, K. et al., 1998. MHC and kin discrimination in juvenile Arctic charr, *Salvelinus alpinus* (L.). *Animal Behaviour*, 56(2), pp.319–327. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9787022.

Ortego, J. et al., 2008. Individual genetic diversity correlates with the size and spatial isolation of natal colonies in a bird metapopulation. *Proceedings of the Royal Society B: Biological Sciences*, 275(1646), pp.2039–47. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2596367&tool=pmcentrez&rendertype=abstract [Accessed March 5, 2015].

Parrott, M.L., Ward, S.J. & Temple-Smith, P.D., 2007. Olfactory cues, genetic relatedness and female mate choice in the agile antechinus (*Antechinus agilis*). *Behavioral Ecology and Sociobiology*, 61(7), pp.1075–1079.

R Core Team, 2014. *R: A Language and Environment for Statistical Computing*, Vienna, Austria. Available at: http://www.r-project.org/.

Rasmussen, L.E.L., Riddle, H.S. & Krishnamurthy, V., 2002. Mellifluous matures to malodorous in musth. *Nature*, 415(February), pp.975–976.

Schaefer, M.L. et al., 2002. Olfactory fingerprints for major histocompatibility complex-determined body odors II: relationship among odor maps, genetics, odor composition, and behavior. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 22(21), pp.9513–9521.

Searcy, W.A. et al., 2015. Geographic Song Discrimination in Relation to Dispersal Distances in

Song Sparrows. *The American Naturalist*, 159(3), pp.221–230.

Setchell, J.M. et al., 2011. Odour signals major histocompatibility complex genotype in an Old World monkey. *Proceedings of the Royal Society B: Biological Sciences*, 278(1703), pp.274–80. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3013381&tool=pmcentrez&rendertype=abstract [Accessed March 5, 2015].

Seutin, G., White, B. & Boag, P.T., 1991. Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology*, 69, pp.82–90.

Taylor, G.T., Haller, J. & Regan, D., 1982. Female rats prefer an area vacated by a high testosterone male. *Physiology and Behavior*, 28, pp.953–958.

Thierry, A., 2014. *Predation risk and the evolution of odours in island birds*. University of Canterbury, Christchurch, New Zealand.

Tregenza, T. & Wedell, N., 2000. Genetic compatibility, mate choice and patterns of parentage: Invited review. *Molecular Ecology*, 9, pp.1013–1027.

Willse, A. et al., 2006. Individual odortypes: interaction of MHC and background genes. *Immunogenetics*, 58(12), pp.967–982. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17089117 [Accessed January 21, 2014].

Wyatt, T.D., 2003. *Pheromones and Animal Behaviour. Communication by Smell and Taste*, Cambridge University Press.

Zavazava, N. & Eggert, F., 1997. MHC and behavior. *Immunology Today*, 18(July), pp.8–10.

Chapter 5

A comparative analysis of preen wax composition between island and continental species of birds

Abstract: *Most birds produce an oily substance called preen wax, which functions in feather maintenance, but may also play a role in sexual selection and chemical communication. It is comprised of a complex mixture of esters and volatiles, and is known to vary in some species with age, sex, season, or environmental conditions. In this study we compared the composition of preen waxes between island birds and their closest continental relatives. We used the birds of the South Pacific region as a model system, focussing on extant passerine species whose evolutionary origin can be traced back to continental Australia, but have since colonized surrounding islands such as New Zealand and New Caledonia (and now constitute isolated endemic species). We collected preen waxes from a total of 162 birds, across 30 species, and compared their chemical profiles via gas chromatography. We found that island birds showed a significant loss of complexity in the non-volatile components but a significant increase in the diversity of compounds in the volatile part of their wax profile. This suggests birds on islands produce preen waxes, and thus odours, that are likely to be more conspicuous than those of continental species. We suggest that, on isolated ecosystems where olfactory-searching predators were absent or scarce, there was little selective pressure against volatile olfactory profiles, and no advantage for birds in suppressing expression of volatile compounds – and hence camouflage their odour - against mammalian or reptilian predators, as could be the case in continental areas. It is possible that this also allowed greater communication through olfactory channels in island birds, and such communication is enhanced through the use of more volatile compounds.*

Introduction

The differences between insular and continental species are a recurrent topic of interest for evolutionary biologists, and islands have acted as real-world laboratories for many studies of biogeography, evolution and speciation (MacArthur & Wilson 1967; Diamond 1974; Gillespie et al. 2008; Losos & Ricklefs 2010). Islands often differ strikingly from continents in their selective environments and, as a result, favour species that evolve in different ways from that of the founding ancestral species. A well-known avian example of evolution and adaptation on islands is that of Darwin's finches on the Galápagos. Darwin hypothesized that the differences in the beak sizes of different species of finches were the result of different food supplies between islands (Darwin, 1859). Later, Mayr (1940) proposed that the reduction in the conspicuousness (colours and songs) of island birds, compared with continental birds, was due to a reduction in interspecific competition. Island species often experience less interspecific overlap on their phenotypes, and thus may face relaxed selection on maintaining isolating mechanisms (e.g., courtship displays, reproductive traits). In turn, this may favour a loss of complexity in the traits that prevent hybridisation (Pfennig & Pfennig 2012).

Another major difference in environmental pressures on islands compared to continents is the generally reduced risk of predation. Some predator guilds are either reduced or absent on islands, due to their reduced capacity to disperse over large oceanic barriers. If colonization is successful, biogeographical theory still posits that the number of species at equilibrium will be lower on an island (MacArthur and Wilson 1967). This effect seems to be stronger for higher trophic levels: it has been theorized, and empirically shown, that often islands lack a top level of the food chain (Terborgh & Estes 2013). Accordingly, mammalian predators are often completely absent from islands (at least before introductions related to human colonisations). This absence of a major predatory guild has allowed specialised phenotypes and life-history traits to evolve in many avian insular species: traits such as the loss of flight, increased body mass, low reproductive rates and naïve behaviours are illustrated by emblematic island species such as the moa, the dodo or the kiwi (Blackburn et al. 2004). Islands can also differ from continental areas in other respects such as climate, habitat, or diversity of parasites, which are also important selective forces driving differences in avian life histories (Martin 1995; Gillespie 2007).

Birds, probably owing in great part to their superior dispersal ability, are a prominent feature of island faunas. Moreover, birds are a convenient model to study evolution: they are conspicuous animals and they have dispersed and undergone speciation on many islands. This chapter examines

the evolution of odours in birds by comparing odorous secretions from island birds and their continental relatives. These secretions originate from the uropygial or preen gland, which is located on the rump and secretes an oily substance called preen wax. Birds collect this oil by squeezing the gland with their bill and then spread it onto their plumage. They spend a considerable amount of time preening everyday, suggesting preen wax has important functions (Cotgreave & Clayton 1994). Although the exact functions of preen wax have been debated, a number of functions have been suggested, including plumage maintenance (Jacob & Ziswiler 1982), water-proofing (Giraudeau et al. 2010), reducing parasite load (Shawkey et al. 2003; Douglas 2008), colour enhancement (López-Rull et al. 2010), predator avoidance (Reneerkens et al. 2005), sexual selection (Zhang et al. 2010), and individual and mate recognition (Whittaker et al. 2010). At least some of these functions are likely to vary in importance for island species, suggesting preen wax of insular birds is likely to be under differing selective pressures than that faced by continental species.

Preen wax is a complex and variable mixture of lipids, esters, fatty acids and alcohols (Jacob & Zisweiler, 1982). It is commonly separated into two fractions, the non-volatile part made of mainly branched long chain esters and the volatile part including compounds such as alkanes and their simple derivatives (Campagna et al. 2012). Wax composition is known to vary across species (Jacob & Ziswiler 1982; Soini et al. 2013; Zhang et al. 2013), populations (Whittaker et al. 2010), and sexes (Mardon et al. 2010; Zhang et al. 2010; Amo, Avilés, et al. 2012; Soini et al. 2013) but is highly repeatable within an individual suggesting a genetic control (Whittaker et al. 2010). It can also be influenced by age or degree of sexual maturity (Sandilands et al. 2004; Shaw et al. 2011), environmental factors (Haribal et al. 2009), and degree of migratory behaviour (Shaw et al. 2011). Some researchers have found preen wax is affected by diet, although this factor is disputed (Thomas et al. 2010; Chapter 3). More recently the parasite community present on the feathers has been suggested to effect the wax make-up of great tits (*Parus major*), with the relative abundance of some compounds changing in response to experimentally modified bacterial loads (Jacob et al. 2014). The composition of preen wax has also been shown in European birds to change at the start of breeding from low molecular weight monoester waxes to high molecular weight diester waxes. Diesters are less volatile than monoesters, thus this switch is proposed to reduce predation by olfactory-searching predators at a time when birds are most vulnerable (Reneerkens et al. 2005; Reneerkens et al. 2006; Soini et al. 2007).

In contrast to continental species, birds that colonised oceanic islands found themselves in habitats with few (or no) predators and less interspecific competition. This difference in evolutionary history between continental and island avifaunas provides an ideal opportunity to study functional interspecific differences in the composition of preen wax. Our project is based in

the South Pacific, a region inhabited by many species whose evolutionary origin can be traced back to continental Australia, where they evolved with a range of predatory and environmental pressures quite different from those on islands. In contrast, the avifaunas on the nearby island archipelagos of New Zealand and New Caledonia evolved in the absence of mammalian predators (until these were introduced by humans) and fewer reptilian predators (e.g., absence of terrestrial snakes). Once established on islands, birds are likely to have developed and expressed evolutionary differences in the composition of their preen waxes. To address this question, we compared preen waxes in paired species between continental Australia, and two island avifaunas from New Zealand and New Caledonia. We expected to see differences in the composition of preen waxes between island birds and closest phylogenetic relatives on continental Australia as a result of the different selection pressures under which birds evolved in each region.

Methods

Preen wax collection

All samples were collected during the breeding season in three locations: in Kaikoura, New Zealand (173°37’E, 42°23’S) from July to December 2012, in Parc des Grandes Fougères, Farino, New Caledonia (165°45’E, 21°37’S) in November 2012 and near Albury, New South Wales, Australia (146°50’E, 36°03’S) in September 2013. All birds were captured by mist-nets, apart from the South Island robin, which was captured by a pull activated drop-trap. A total of 162 birds of 30 species were captured and had their preen waxes collected and analysed by gas chromatography: 33 birds of 5 species in New Zealand, 63 birds of 12 species in New Caledonia and 66 individuals of 13 species in Australia (Table 5.1). All species were native or endemic to their respective area. Each bird was banded or marked to avoid resampling the same individual twice.

Preen wax was obtained by gently pressing the uropygial gland and collecting a small drop of wax with a sterile metal loop. This loop was immediately transferred into a sterile 100 µl glass insert and the insert was then placed inside a sterile glass vial. To minimise contamination, all glassware and loops had previously been cleaned with a sequence of 3 organic solvents, baked dry, and stored in clean vials until used. Samples were stored in a cooler for a few hours while in the field and then later frozen at -20°C until analysed. All birds and equipment were handled while wearing disposable rubber gloves to avoid contaminating samples with human odours.

Table 5:1: List of birds used in this study. The number of individuals sampled is given in the total column. Species number follows those given in Figures 5.1 and 5.3

Species N°	Common name	Scientific name	Total
New Zealand			
1	Bellbird	*Anthornis melanura*	6
2	Grey fantail	*Rhipidura fuliginosa*	7
3	Grey warbler	*Gerygone igata*	6
4	Silvereye	*Zosterops lateralis*	6
5	South Island robin	*Petroica australis*	8
New Caledonia			
6	Barred honeyeater	*Phylidonyris undulatus*	1
7	Dark-brown honeyeater	*Lichmera incana*	6
8	New Caledonian flycatcher	*Myiagra caledonica*	3
9	Fan-tailed gerygone	*Gerygone flavolateralis*	5
10	Grey fantail	*Rhipidura albiscapa bulgeri*	2
11	Streaked fantail	*Rhipidura verreauxi*	9
12	Red-throated parrotfinch	*Erythrura psittacea*	3
13	Rufous whistler	*Pachycephala rufiventris xanthetraea*	3
14	New Caledonian whistler	*Pachycephala caledonica*	6
15	Silvereye	*Zosterops lateralis griseonata*	11
16	Green-backed white eye	*Zosterops xanthochrous*	12
17	Yellow-bellied robin	*Microeca flaviventris*	2

Species N°	Common name	Scientific name	Total
Australia			
18	Fuscous honeyeater	*Lichenostomus fuscus*	5
19	White-plumed honeyeater	*Lichenostomus penicillatus*	6
20	Yellow-tufted honeyeater	*Lichenostomus melanops*	6
21	Western gerygone	*Gerygone fusca*	1
22	Yellow thornbill	*Acanthiza nana*	3
23	White-browed scrubwren	*Sericornis frontalis*	7
24	Grey fantail	*Rhipidura albiscapa alisteri*	8
25	Willie wagtail	*Rhipidura leucophrys*	6
26	Rufous whistler	*Pachycephala rufiventris rufiventris*	2
27	Red-browed finch	*Neochmia temporalis*	6
28	Diamond firetail	*Stagonopleura guttata*	1
29	Silvereye	*Zosterops lateralis*	6
30	Eastern yellow robin	*Eopsaltria australis*	3

Gas chromatography analysis

Each preen wax sample was mixed, within its glass insert, with 100 µl of ethyl acetate. This mix was then vortexed for 60 seconds at 700 rpm to ensure proper dissolution between the solvent and the wax. A sample of 1 µl was then injected in the gas chromatographer (GC) with a split ratio of 6:1. The GC was a Shimadzu GC-2010, equipped with a Shimadzu AOC20i+s auto-injector and a Varian CP-SIL 5 CB capillary column (25 m length x 320 µm internal diameter x 0.12 µm film thickness). The injection port temperature was set at 250°C, the carrier gas was Nitrogen with a total flow of 19.0 ml/min and a linear velocity of 36.7 cm/sec. The FID detector operated at 320°C, with a sampling interval of 40 msec. Oven temperature was programmed with an initial temperature of 70°C and a hold time of 4 mins, then an increase to 130°C at a rate of 20°C/min, and finally an increase to 320°C at a rate of 4°C/min and a hold time of 15 minutes.

Chromatograms (Figure 5.1) were obtained with the software Shimadzu's GCSolution, version 2.3 (©Shimadzu 2002-2009). We focused first on the nonvolatile fraction of the profile (wax esters) as described in Reneerkens, Piersma, & Damsté (2005). From each GC profile, we recorded 4 data types: mean retention time (RT), RT range, minimum RT and number of peaks. As we could not control for the original amount of wax collected from each bird, we used the relative area of each peak and calculated a weighed mean RT (RT * relative peak area), hereafter just stated as mean RT. The RT range corresponded to the time between the first peak (minimum RT) and the last peak of the wax ester fraction. Finally, the number of peaks was calculated for the wax ester part.

At this stage we could not identify the chemical composition of each peak as our samples still have to be processed through a mass spectrometer, therefore multivariate comparison of the

traces was not feasible at the inter-specific level. In other words, as we could not identify the specific compound(s) responsible for each peak, we cannot be confident that peaks eluting at the same retention time correspond to the same molecule, especially if they come from samples belonging to different species. Nevertheless, for the purposes of this study we were more interested in relative differences in the volatilities of preen wax between island and continental species. As the retention times produced by the gas chromatograph provides a useful surrogate of volatility (i.e., heavier molecular weight and therefore less volatile compounds exhibit higher retention times), comparisons of retention time can provide an initial test of any differences in the volatility of preen waxes between island and continental birds.

Next, we visually counted the number of peaks present between the solvent peak and the start of the wax ester fraction; this is referred to as the "volatile fraction" (Figure 5.1). We could not analyse as many variables as for the "ester fraction" because our GC settings were optimised for detecting the wax esters and thus may have missed some of the more volatile molecules in the "volatile fraction".

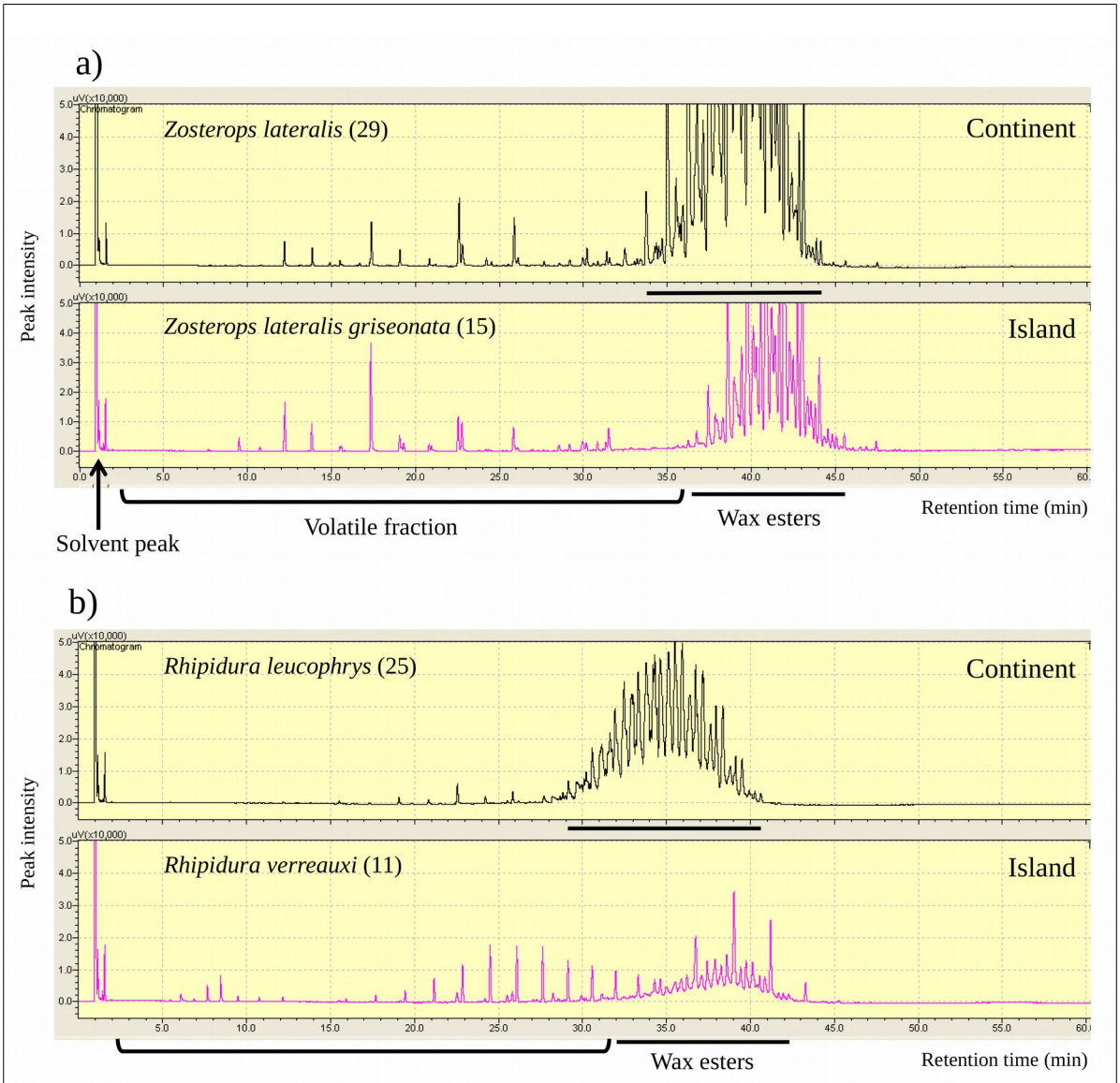

Figure 5.1: Example of chromatograms from two pairs of closely related bird species: a) silvereye pair and b) fantail pair. Species numbers follow those given in Table 5.1 and Figure 5.3. In both species pairs, the island species has more peaks in the "volatile" region but fewer in the "wax ester" region.

Statistical analysis

All data followed a normal distribution. A two-way ANOVA was performed with sex and geographic location (island or continent) as factors. Dependent variables were mean RT, RT range, minimum RT and number of peaks for the ester fraction, and the number of peaks for the volatile fraction. We first considered all the dependent variables detailed above jointly, and entered them in a MANOVA analysis: as this detected significance for geographic location, we proceeded with separate ANOVAs to ascertain which of the dependent variables were influenced by geographic location. A series of one-way ANOVAs, with male and female data pooled together was then performed with the geographic location as factor and the same dependent variables.

To determine if any differences in the number of peaks between island and continental birds were confounded by phylogenetic effects, we used the pair-wise method (Moller & Birkhead 1992). We selected 8 closely related species pairs of which 6 were paired congeners (Table 5.1 and Figure 5.3). We used recent phylogenetic trees to select the closest related species (Arnaiz-Villena et al. 2009; Christidis et al. 2011) and compared them with a paired t-test.

Finally, we performed a one-way ANOVA with the visually counted number of peaks from the volatile fraction. Data for the pair-wise comparison was not normally distributed, even after transformation, so a Wilcoxon matched pairs test was used. All statistical analyses in this study used the programme STATISTICA 6.0, © StatSoft Inc. The statistical significance level was $P = 0.05$.

Results

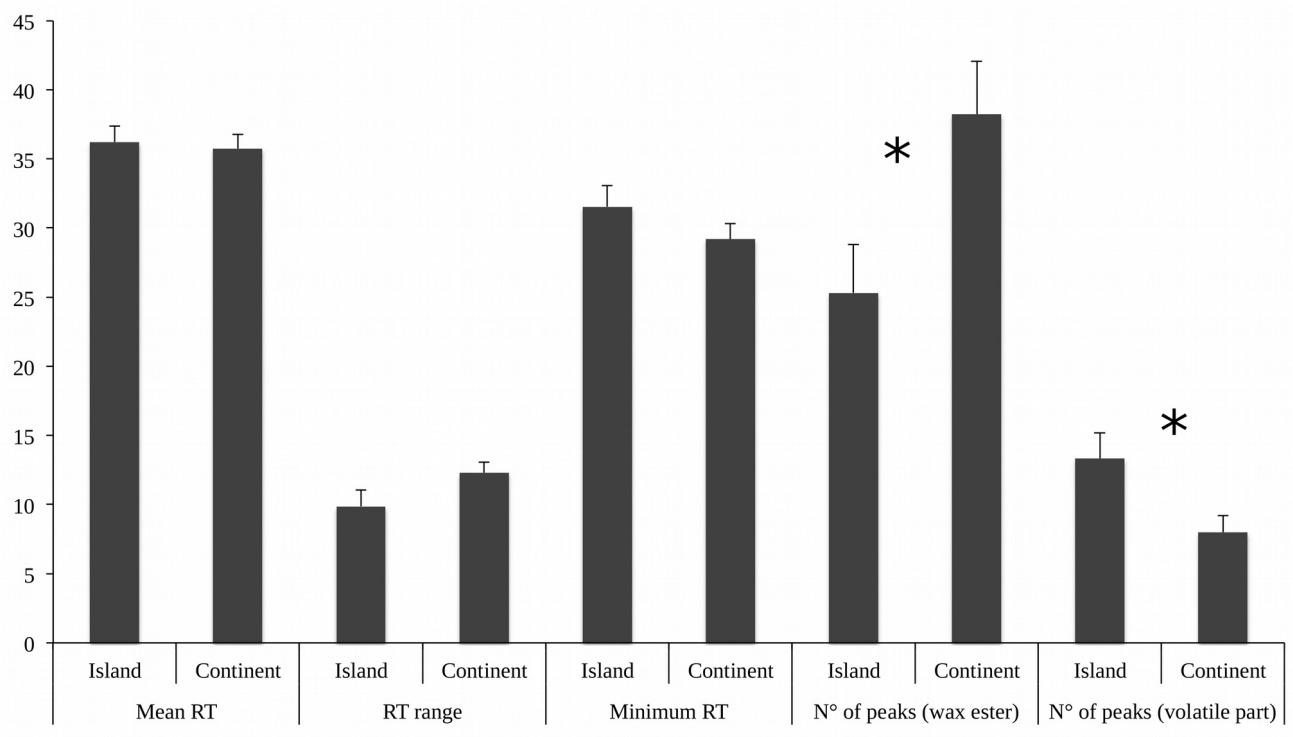

Figure 5.2: Averages of weighed mean retention time (RT), RT range, minimum RT and number of peaks obtained from chromatograms of 162 birds. Island birds show a significant lower number of peaks in their wax ester fraction and a significant higher number of peaks in their volatile fraction compared with continental species. RT is measured in minutes. Number of peaks is the average count of peaks in the two fractions of the gas chromatographic profile.

Ester fraction

A two-way MANOVA showed no effect of sex (sex: Wilks' $\lambda_{12,\ 43.9} = 0.14$, $P = 0.97$) and no significant interaction between sex and the geographic location (interaction: Wilks' $\lambda_{12,\ 43.9} = 0.59$, $P = 0.67$). However, a significant effect of the geographic location was found (status: Wilks' $\lambda_{12,\ 43.9} = 9.51$, $P < 0.001$). We therefore divided this test into 4 separate two-way ANOVAs and found a significant effect of geographic location (island or continent) for the number of peaks ($F_{1,\ 43} = 14.79$, $P < 0.001$) and the RT range (F (1, 43) = 12.18, $P < 0.01$). The mean RT (F $F_{1,\ 43} = 0.10$, $P = 0.75$) was non-significant but minimum RT showed a non-significant trend towards a slightly lower RT in the continental species (F $F_{1,\ 43} = 3.63$, $P = 0.063$). Overall, island species had fewer peaks and a narrower range of RT in the ester fraction.

Since there was no effect of sex, we pooled data from male and female together for each variable and confirmed that island birds had a significantly lower number of peaks in their wax ester fraction than continental species (one-way ANOVA: $F_{1,\ 29} = 6.18$, $P = 0.02$; Figures 5.1 and 5.2). The minimum RT ($F_{1,\ 29} = 1.49$, $P = 0.23$) and mean RT ($F_{1,\ 29} = 0.09$, $P = 0.77$) were non-significantly different between insular and continental birds, while RT range showed a non-significant trend ($F_{1,\ 29} = 2.96$, $P = 0.09$) (Figure 5.2). When controlled for phylogenetic effects, island species of birds had a lower number of peaks in their wax ester fraction compared with continental species (Figure 5.3, paired t-test: t (7) = -2.42, $P = 0.046$).

Volatile fraction

In contrast to the ester fraction, island birds showed a significantly higher number of peaks in their volatile part of the GC profile compared with continental birds (one-way ANOVA: $F_{1,\ 28} = 5.48$, $P = 0.03$; Figures 5.1 and 5.2). When controlled for phylogenetic effects, island birds showed a marginally non-significant trend towards a higher number of peaks in the volatile part compared with continental species (Wilcoxon test: Z = 1.86, $P = 0.06$).

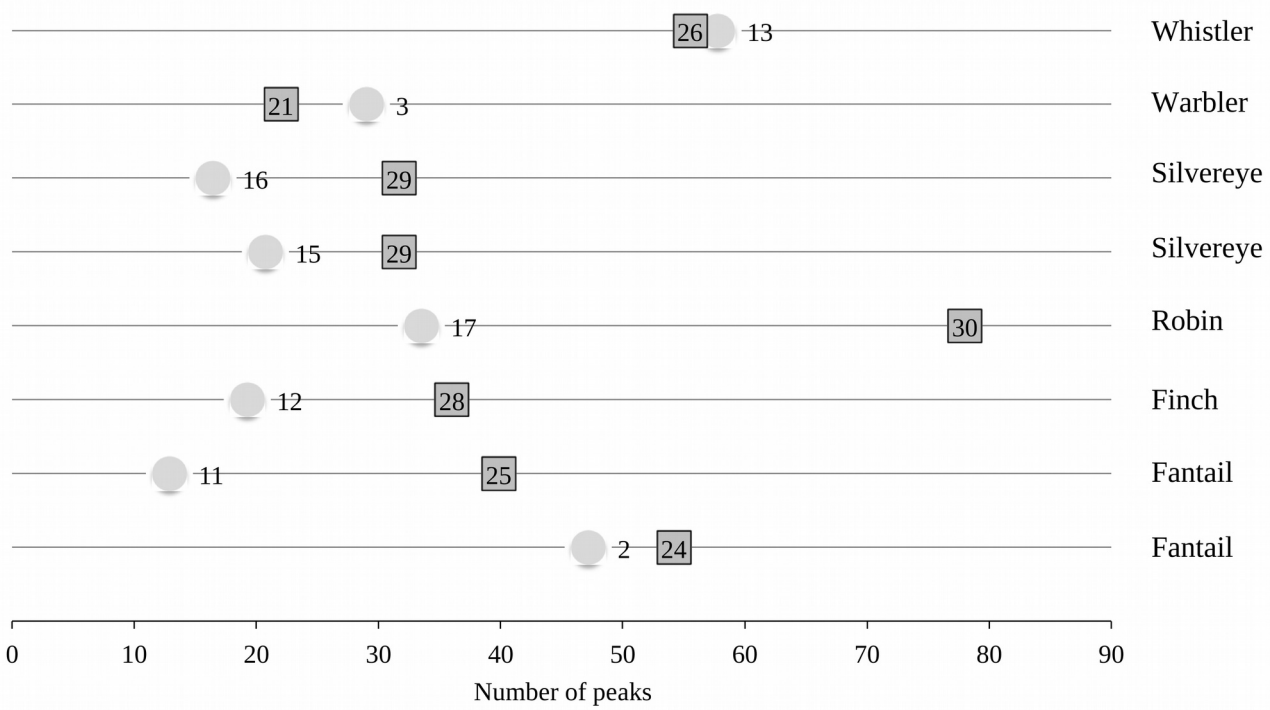

Figure 5.3: Paired comparison of the number of peaks from the wax ester fraction, between 8 closely related species of island (circles) and continental (squares) birds. For clarity purposes, species are represented by numbers and exact names are detailed in Table 5.1.

Discussion

Our comparison of preen wax composition in a range of passerine birds between continental Australia and the two island avifaunas on New Zealand and New Caledonia revealed consistent directional differences. In general, island species of birds produced preen waxes with fewer components in the ester fraction but more components in the volatile fraction. This overall shift towards lighter molecular weight compounds is likely to result in the preen waxes of island birds being more conspicuous, as volatile components are easier to detect. This result held when we controlled for potential phylogenetic effects as the pattern remained when we only compared closely related species in which one member of a pair evolved in continental Australia while the other evolved on an island. Why should island birds produce preen wax that differs from that of continental birds?

The decreased number of peaks in the ester fraction in island birds indicates a loss of complexity in the less volatile lipidic components of the wax. One potential explanation could be the impoverished parasite communities found on islands (de Bellocq et al. 2003; Magnanou & Morand 2006). Preen wax has been shown to inhibit the growth of feather-degrading bacteria (Shawkey et al. 2003; Reneerkens et al. 2008), and it seems to have some effect on the feathers'

invertebrate ectoparasite community as well (Moyer et al. 2003): fewer bacterial and/ or invertebrate parasites in island birds (in both numbers of individuals as well as diversity of species) may mean that birds require fewer types of “long lasting” lipids to maintain their feathers. It is not clear whether a greater molecular diversity of esters is needed to control a broader range of ectoparasites, but it seems plausible that simpler parasite faunas may not need to be countered by as many chemical “weapons” in the form of preen wax esters. We were unable to survey the ectoparasite faunas of each species, although we did not find any difference in the prevalence of feather mites between island and continental species (unpubl. data).

Another potential explanation for the difference in preen wax between continental and island birds is that the intensity of sexual selection may be reduced in insular populations (Friedman et al. 2009; Price et al. 2009) If preen wax functions as a sexual signal between individuals (as suggested in one of our surveyed species by Thierry 2014), then fewer types of compounds may be needed by island birds under relaxed sexual selection. Island birds also show a general loss of colours and plumage brightness (Doucet et al. 2004; Thomas et al. 2014). The reasons for this loss are unclear, but with reduced plumage colour and ornaments there may be a decreased need for preen waxes to maintain such feathers. A generally darker plumage is known to reduce feather abrasion and protect against the damage from UV radiations (Burtt 1986) but it is unknown whether melanistic feathers require a different preen wax for their maintenance. It is possible that a smaller variety of lipids are needed to maintain feathers with duller colours. With a decrease in sexual selection on islands, birds may have a decreased need for preen waxes. However, as reported by Thierry (2014), island birds have bigger uropygial glands and as a result probably produce larger volumes of preen waxes than their continental counterparts. This argues against a decreased need for preen wax due to reduced plumage ornaments. A decrease in preen wax complexity due to a reduced signalling function also seems unlikely, as although it is true that the ester fraction is consistent with this hypothesis, the increase in the volatile fraction suggests the opposite, with island birds producing more volatile components in their preen wax and at least the potential for an increased signalling function.

As birds tend to lose colours and show reduced complexity of songs on islands (Friedman et al., 2009; Price et al., 2009), sexual selection may favour a different pathway to assess potential mates, and therefore favour the use of olfactory signals as a channel of communication. This trade off between visual/auditory signals and olfactory signals might explain the presence of more volatile preen waxes. Such olfactory communication would have only been possible on islands because the predation risk by olfactory searching predators was non-existent or considerably reduced (at least until predatory mammals were introduced by humans). This trade off would have been particularly favoured for species living in a dense and dark forest where visual cues are

obscured (e.g. South Island robin). For example, odours could be used to identify conspecifics trying to enter a territory, or even used to mark territorial boundaries. On continental areas, such a function of preen wax would be constrained by the increased risk of predation by predatory mammals using olfactory cues to locate their prey. Whether islands have "liberated" birds from the olfactory constraints faced by the continental relatives is not yet known, but would certainly be a worthy and interesting area of investigation. Results from experiments carried out by my group (Thierry 2014) on South Island robins are indeed consistent with this hypothesis.

Although we found differences between island and continental species of species of birds, we found no significant difference in the composition of preen waxes between the sexes. This result contrasts to what has been found in some other studies in which males can have different compounds in their preen wax compared to females (Zhang et al. 2010; Amo, López-Rull, et al. 2012; Soini et al. 2013). It is possible we did not find a difference as we only examined general features of the GC profile (mean retention time, RT range, minimum RT, number of peaks) and could not identify each of the individual compounds that comprised each peak. This issue will be examined once our samples are processed through a mass spectrometer. Nevertheless, the similarity and high degree of overlap in the peaks between males and females suggests sexual differences are likely to be small, especially in comparison to that observed between species and geographic locations.

We were careful to limit other factors confounding our results, such as degree of sexual maturity or season by only sampling adult birds during the breeding season. We also controlled for migration or environmental effects by only sampling non-migratory forest passerines captured from locations with similar altitudes and latitudes. Our Australian field site is indeed situated in between the two islands with a difference of 15°S with New Caledonia and a difference of 6°S with New Zealand (see introduction for exact GPS coordinates). Although some authors have found an effect of diet on the composition of preen waxes (Thomas et al. 2010), we have not found support for this in my own experiment conducted on silvereyes *Zosterops lateralis*, a species present both in Australia and New Zelaand (silvereyes self-introduced to New Zealand in recent times, see Chapter 3) and included in our comparative study.

It is important to note that the intensity of an odour is proportional to the quantity of: a) the odorous secretion exuded, and b) the absolute concentration of compounds within the secretion, but the identity of the odour profile is created by the quantitative (within species) and qualitative (between species) variation within the "bouquet" of compounds (Zhang et al. 2009; Bonadonna & Mardon 2013). This is consistent with the chemical profiles codified by epicuticular hydrocarbons in insects: in the primitively eusocial *Polistes* wasps, for instance, cuticular blends from conspecific

individuals contain the same compounds (among colony differentiation being driven by quantitative differences in the profile, i.e. variation in the relative proportions of compounds), while among species variation is driven by qualitative differences, i.e. presence/ absence of compounds (Lorenzi 2006). Until GC-MS analysis is complete, it is not possible for us to confirm if the species analysed in this study follow the same pattern – it has been confirmed, though, that within-species variation is quantitative in nature: barring seasonal shifts, conspecific profiles are made up of the same compounds.

It will be essential to integrate the information coming from mass-spectrometric analysis of the samples, but this preliminary suite of univariate tests has highlighted interesting differences in the preen wax profile of continental *vs.* island species, which is consistent with the different selective pressures that these species experience in the different ecosystems.

References

Amo, L., López-Rull, I., et al., 2012. Male quality and conspecific scent preferences in the house finch, *Carpodacus mexicanus*. *Animal Behaviour*, 84, pp.1483–1489.

Amo, L., Avilés, J.M., et al., 2012. Sex recognition by odour and variation in the uropygial gland secretion in starlings. *The Journal of animal ecology*, 81(3), pp.605–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22220811 [Accessed September 25, 2014].

Arnaiz-Villena, A., et al., 2009 Estrildinae finches (Aves, Passeriformes) from Africa, South Asia and Australia: a molecular phylogeographic study. *The Open Ornithology Journal*, 2(1), 29-36

De Bellocq, J.G. et al., 2003. A comparison of the structure of helminth communities in the woodmouse, *Apodemus sylvaticus*, on islands of the western Mediterranean and continental Europe. *Parasitology research*, 90(1), pp.64–70.

Blackburn, T.M. et al., 2004. Avian Extinction and Mammalian Introductions on Oceanic Islands. *Science (New York, N.Y.)*, 305, pp.1955–57.

Bonadonna, F. & Mardon, J., 2013. Besides Colours and Songs, Odour is the New Black of Avian Communication. In M. L. East & M. Dehnhard, eds. *Chemical Signals in Vertebrates 12*. Springer New York, pp. 325–339. Available at: http://dx.doi.org/10.1007/978-1-4614-5927-9_26.

Burtt, E.H., 1986. An analysis of physical, physiological, and optical aspects of avian coloration with emphasis on wood-warblers. *Ornithological Monographs*, 38(38), pp.1–126.

Campagna, S. et al., 2012. Potential semiochemical molecules from birds: a practical and comprehensive compilation of the last 20 years studies. *Chemical senses*, 37(1), pp.3–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21798850 [Accessed January 21, 2014].

Christidis, L. et al., 2011. Mitochondrial and nuclear DNA phylogenies reveal a complex evolutionary history in the Australasian robins (Passeriformes: Petroicidae). *Molecular phylogenetics and evolution*, 61(3), pp.726–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21867765 [Accessed September 28, 2014].

Darwin, C., 1859 *On the origin of species by means of natural selection.* London, Murray

Cotgreave, P. & Clayton, D.H., 1994. Comparative analysis of time spent grooming by birds in

relation to parasite load. *Behaviour*, 131(4), pp.171–187.

Diamond, J.M., 1974. Colonization of exploded volcanic islands by birds: the supertramp strategy. *Science*, 184(4138), pp.803–806.

Doucet, S.M. et al., 2004. Concordant evolution of plumage colour, feather microstructure and a melanocortin receptor gene between mainland and island populations of a fairy-wren. *Proceedings of the Royal Society B: Biological Sciences*, 271(1549), pp.1663–1670. Available at: http://rspb.royalsocietypublishing.org/cgi/doi/10.1098/rspb.2004.2779.

Douglas, H.D., 2008. Prenuptial perfume: Alloanointing in the social rituals of the crested auklet (*Aethia cristatella*) and the transfer of arthropod deterrents. *Naturwissenschaften*, 95(1), pp.45–53.

Friedman, N.R. et al., 2009. Correlated Evolution Of Migration And Sexual Dichromatism In The New World Orioles (Icterus). *Evolution*, 63(12), pp.3269–3274. Available at: http://doi.wiley.com/10.1111/j.1558-5646.2009.00792.x.

Gillespie, R.G., 2007. Oceanic Islands: Models of diversity. *Encyclopedia of Biodiversity*, pp.1–36, Elsevier.

Gillespie, R.G., Claridge, E.M. & Goodacre, S.L., 2008. Biogeography of the fauna of French Polynesia: diversification within and between a series of hot spot archipelagos. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 363(September), pp.3335–3346.

Giraudeau, M. et al., 2010. Effect of restricted preen-gland access on maternal self maintenance and reproductive investment in mallards. *PloS one*, 5(10), p.e13555. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2965083&tool=pmcentrez&rendertype=abstract [Accessed January 21, 2014].

Haribal, M., Dhondt, A. & Rodriguez, E., 2009. Diversity in chemical compositions of preen gland secretions of tropical birds. *Biochemical Systematics and Ecology*, 37(2), pp.80–90. Available at: http://dx.doi.org/10.1016/j.bse.2008.12.005.

Jacob, J. & Ziswiler, V., 1982. Chapter 4 - THE UROPYGIAL GLAND. In D. Farner, J. King, & K. Parkes, eds. *Avian Biology*. Amsterdam: Academic Press, pp. 199–324. Available at: http://www.sciencedirect.com/science/article/pii/B9780122494062500137.

Jacob, S. et al., 2014. Uropygial gland size and composition varies according to experimentally modified microbiome in Great tits. *BMC Evolutionary Biology*, 14, p.134. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4074404&tool=pmcentrez&rendertype=abstract [Accessed September 15, 2014].

López-Rull, I., Pagán, I. & MacÍas Garcia, C., 2010. Cosmetic enhancement of signal coloration: Experimental evidence in the house finch. *Behavioral Ecology*, 21(May), pp.781–787.

Lorenzi, M., 2006. The result of an arms race: the chemical strategies of *Polistes* social parasites. *Annales Zoologici Fennici*, (December), pp.550–563. Available at: http://www.sekj.org/PDF/anz43-free/anz43-550.pdf.

Losos, J. & Ricklefs, R., 2010. *The Theory of Island Biogeography Revisited*,

MacArthur, R.H. & Wilson, E.O., 1967. *The theory of island biogeography*, Princeton University Press.

Magnanou, E. & Morand, S., 2006. Insularity and micromammal-macroparasite relationships. In S. Morand, B. Krasnov, & R. Poulin, eds. *Micromammals and Macroparasites*. Tokyo: Springer Japan, pp. 295–315. Available at: http://dx.doi.org/10.1007/978-4-431-36025-4_16.

Mardon, J. et al., 2010. Species, gender, and identity: cracking petrels' sociochemical code. *Chemical senses*, 35(4), pp.309–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20190009 [Accessed September 28, 2014].

Martin, T.E., 1995. Avian Life History Evolution in Relation to Nest Sites, Nest Predation, and Food. *Ecological Monographs*, 65(1), pp.101–127.

Mayr, E., 1940. Speciation phenomena in birds. *The American Naturalist*, 74(752), 249-278.

Moller, A.P. & Birkhead, T.R., 1992. A Pairwise Comparative Method as Illustrated by Copulation Frequency in Birds. *The American Naturalist*, 139(3), p.644.

Moyer, B.R., Rock, A.N. & Clayton, D.H., 2003. Experimental test of the importance of preen oil in rock doves (*Columba livia*). *The Auk*, 120(2), pp.490–496. Available at: C:\Users\Gar\Documents\My Dropbox\Bibliografia\PDF\Moyer2003.pdf.

Pfennig, D.W. & Pfennig, K.S., 2012. *Evolution's wedge: competition and the origins of diversity*, Univ of California Press.

Price, J.J., Lanyon, S.M. & Omland, K.E., 2009. Losses of female song with changes from tropical to temperate breeding in the New World blackbirds. *Proceedings of the Royal Society B: Biological Sciences*, 276(1664), pp.1971–1980. Available at: http://rspb.royalsocietypublishing.org/cgi/doi/10.1098/rspb.2008.1626.

Reneerkens, J. et al., 2008. Seasonally Changing Preen-wax Composition: Red Knots' (Calidris canutus) Flexible Defense against Feather-degrading Bacteria? T. PIERSMA & E. H. BURTT, eds. *The Auk*, 125(2), pp.285–290. Available at: http://www.bioone.org/doi/abs/10.1525/auk.2008.06217 [Accessed September 8, 2015].

Reneerkens, J., Piersma, T. & Damsté, J.S.S., 2006. Discerning adaptive value of seasonal variation in preen waxes: comparative and experimental approaches. *Acta Zoologica Sinica*, 52, pp.272–275.

Reneerkens, J., Piersma, T. & Damsté, J.S.S., 2005. Switch to diester preen waxes may reduce avian nest predation by mammalian predators using olfactory cues. *The Journal of experimental biology*, 208, pp.4199–4202.

Sandilands, V. et al., 2004. Preen gland function in layer fowls: factors affecting preen oil fatty acid composition. *British poultry science*, 45(1), pp.109–115. Available at: http://www.tandfonline.com/doi/abs/10.1080/00071660410001668932 [Accessed January 21, 2014].

Shaw, C.L. et al., 2011. Volatile and Semivolatile Compounds in Gray Catbird Uropygial Secretions Vary with Age and Between Breeding and Wintering Grounds. *Journal of Chemical Ecology*, 37, pp.329–339.

Shawkey, M.D., Pillai, S.R. & Hill, G.E., 2003. Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. *Journal of Avian Biology*, 34(4), pp.345–349. Available at: C:\Users\Gar\Documents\My Dropbox\Bibliografia\PDF\Shawkey2003b.pdf.

Soini, H.A. et al., 2013. Chemosignaling diversity in songbirds : Chromatographic profiling of preen oil volatiles in different species. *Journal of Chromatography A*, 1317, pp.186–192. Available at: http://dx.doi.org/10.1016/j.chroma.2013.08.006.

Soini, H.A. et al., 2007. Seasonal Variation in Volatile Compound Profiles of Preen Gland Secretions of the Dark-eyed Junco *(Junco hyemalis*). *Journal of Chemical Ecology*, 33, pp.183–198.

Terborgh, J. & Estes, J.A., 2013. *Trophic Cascades: Predators, Prey, and the Changing Dynamics*

of Nature, Island Press. Available at: https://books.google.co.nz/books?id=tjOT8KJ6mF8C.

Thierry, A., 2014. *Predation risk and the evolution of odours in island birds*. University of Canterbury, Christchurch, New Zealand.

Thomas, D.B. et al., 2014. Non-destructive descriptions of carotenoids in feathers using Raman spectroscopy. *Analytical Methods*, 6(5), pp.1301–1308.

Thomas, R.H. et al., 2010. Use of TLC-FID and GC-MS/FID to examine the effects of migratory state, diet and captivity on preen wax composition in White-throated Sparrows *Zonotrichia albicollis*. *Ibis*, 152, pp.782–792.

Whittaker, D.J. et al., 2010. Songbird chemosignals: volatile compounds in preen gland secretions vary among individuals, sexes, and populations. *Behavioral Ecology*, 21(3), pp.608–614. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2854530&tool=pmcentrez&rendertype=abstract [Accessed January 21, 2014].

Zhang, J. et al., 2010. Uropygial Gland-Secreted Alkanols Contribute to Olfactory Sex Signals in Budgerigars. , pp.375–382.

Zhang, J., Sun, L. & Zuo, M.-X., 2009. Uropygial gland volatiles may code for olfactory information about sex, individual, and species in Bengalese finches *Lonchura striata*. *Current Zoology*, 55(5), pp.357–365.

Zhang, J.-X. et al., 2010. Uropygial gland-secreted alkanols contribute to olfactory sex signals in budgerigars. *Chemical senses*, 35(5), pp.375–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20212012 [Accessed July 28, 2014].

Zhang, Y.-H., Du, Y.-F. & Zhang, J.-X., 2013. Uropygial gland volatiles facilitate species recognition between two sympatric sibling bird species. *Behavioral Ecology*, 00, pp.1–8.

Chapter 6

General discussion

Avian ecology, biology and physiology have been the subject of much research, and birds have a long and successful history as model organisms, having been central to the development of great scientific works starting with Darwin and Wallace's *On the Tendency of Species to form Varieties; and on the Perpetuation of Varieties and Species by Natural Means of Selection* (1858). The reason why birds were prominently featured in the work of many early naturalists is probably at least partly due to the ease with which they can be observed – at least compared to other, more secretive animal groups – and to the strikingness (at least to the human senses) of their visual and acoustic displays. This very detail, and the concurrent fact that birds rarely display any overt olfactory-driven behaviours such as sniffing or scent-marking (Bonadonna and Mardon 2013), has resulted in a general neglect of the chemical modality of communication within the body of avian research. However, this oversight has changed during the past decade, and there is now a growing number of articles published on avian olfactory abilities, odour emission and chemical communication (see Figure 2.1, Chapter 2). Indeed, most aspects of avian life, from navigation, to foraging, to kin recognition, mate selection and protection against predators and parasites have started to be analyzed in the light of chemical ecology and potential role that sociochemicals may play. Nevertheless, there is still a lot to do, and this field of research is still in its infancy, at least compared to that undertaken in insects and other invertebrates. It was my primary objective to fill in some of the gaps in our understanding of chemical signals in birds with the research presented in this thesis.

In chapter 2, I analyzed the production and composition of preen wax in a variety of nestling birds, which are often overlooked in studies on production of chemical cues. This was an exploratory study, due to the novelty of the research, but I hypothesized the existence of a more

volatile and precocious chemical profile for nestlings of native species compared to nestlings of introduced species, due to their differing evolutionary histories with olfactory-searching predators. This was not supported by my data, primarily because of the peculiar profile displayed by European starlings, which start producing preen wax very early on and show a great number of eluting peaks compared to all other species. Nevertheless, the multivariate analysis of the profiles still showed a significant difference among native and introduced species, which indicates that the two assemblages might indeed differ in some aspect of their multivariate profile. At this early stage, it is difficult to draw conclusions as a much larger number of species need to be analysed before robust statistical comparisons can be made. However, the variation I observed between species, and over the period while young are in the nest, suggests a number of future avenues for research. For example, what are the functions of preen wax in nestling birds, and are they the same as in adults? Are preen waxes used in parent-offspring communication, and if so, how does this trade-off with other avenues of communication such as acoustic begging calls and behavioural posturing? Perhaps adults might even use odour signals from their offspring to assess their condition, and in species with brood reduction, use this as a cue when adjusting levels of parental care.

In chapter 3, I experimentally tested the effect of a change in diet on preen wax composition in a generalist species widespread in the South Pacific, the silvereye *Zosterops lateralis*. This was an important question for me to investigate, as I realized that the conclusions previously reached in many chemical ecology studies which investigated differences in preen wax composition between individuals of different ages (Amo et al. 2012), breeding stages (Reneerkens et al. 2002), species (Haribal et al. 2009), or occupying different habitats (Bonadonna and Bretagnolle 2002) could have been confounded by the potential influence of diet on preen wax profiles, which has not been unequivocally or satisfactorily clarified. The limited biochemical understanding we have of the preen wax production process seems to discount such direct links between dietary intake and uropygial gland composition (Noble et al. 1963), but the one study that experimentally tested the effect of diet on chemical profiles in one passerine species did find a significant effect (Thomas et al. 2010). I wanted to contribute to the field by performing a diet supplementation experiment in the wild, with a set-up that might be more relevant for ascertaining whether different diets could hamper studies that are investigating other main drivers of chemical diversity. My results were clear: I found that diet does not seem to have an effect on the profiles of silvereyes sampled during a moth long experiment in which they were given access to fat. This suggests that other aspects of my study (and perhaps much of the literature) are not confounded by dietary effects, although caution is still needed in this assumption as it would be valuable to test further species, and those with differing or more specialized diets.

In chapter 4, I investigated the influence of inbreeding and geographic isolation on the chemical profiles of a native New Zealand species, the South Island Robin *Petroica australis*. There are several indications that avian chemical signals are endogenous in nature (Hirao et al. 2009; Mardon et al. 2010; Célérier et al. 2011; Leclaire et al. 2012; Leclaire et al. 2014) and it is interesting to know if uropygial gland secretions can encode information on genetic relatedness, levels of genetic diversity and the degree of genetic compatibility of conspecifics. Having access to an island where I could collect samples from offspring of isolated, bottlenecked individuals and offspring of "outcrossed" pairs, I have been able to test whether such information was present at the chemical level or not. My results indicated that the robins' profiles did allow for discrimination of the inbred *vs.* outbred birds, but the same chemical profiles did not seem to correlate with heterozygosity levels, as measured by microsatellites. While microsatellites have been extensively used to quantify global (i.e. whole-genome) genetic diversity, they are neutral markers and do not code for any trait, much less any trait related to chemical signals. As it has been pointed out already (Crandall et al. 2000; McKay and Latta 2002; Holderegger et al. 2006), microsatellites might therefore not always be the most useful tool for the type of analyses I used in my study, and correlates of adaptive genetic diversity, especially if related to the trait of interest, might be more appropriate. In this case, a promising way forward would be to repeat the analysis by measuring diversity at the MHC loci, which is linked to olfactory discrimination and mate choice in many mammals (see Tregenza and Wedell 2000 for a review) and has lately started to be investigated in birds as well (Zelano and Edwards 2011; Leclaire et al. 2014). Nevertheless, such more in depth lab analyses should not be done in isolation, and field studies are needed to simultaneously test whether the differences detected in preen wax profiles are actually used by the birds in social communication. If they are used in key life history decisions, such as mate choice, then the signals encoded in preen wax variation may be important when making decisions for captive breeding of endangered species. A study by Thierry (2014) provided some evidence that South Island robins can detect the preen wax by its odour alone, suggesting the potential for individual signals to be coded in the preen wax profile, which may in turn be reflective of individual genetic attributes.

Finally, in chapter 5, in collaboration with Aude Thierry, we compared profiles of island *vs.* continental species pairs, to uncover any pattern of chemical signal evolution across different selective landscapes. We chose this set-up to be able to comparatively test the hypothesis that the absence of olfactory-searching predators on some avifaunas can lead to the expression of more diverse or more volatile profiles compared to what is allowed in an environment that is heavily populated with olfactory-searching predators. We have found this to be the case, with island birds possessing higher peak richness in the volatile part of the preen wax profile, while at the same time

showing fewer compounds in the heavy, ester dominated fraction of the chromatogram. The heavier fraction of the preen wax could be very positively selected, and maintained, as “olfactory camouflage” (Reneerkens 2005) in Australia, where mammals and snakes abound (and which are both absent in New Zealand and New Caledonia). It is possible that production of heavier molecules is costlier for the birds, in which case the lower predatory pressure in New Zealand and New Caledonia would not only allow more volatile profiles to be displayed – and possibly used in communication, see Thierry, 2015 – but also remove any advantage of maintaining the heavier fraction in the preen wax, in accordance with our hypothesis. Further predator bioassays – in addition to hose conducted by Thierry (2015) – may contribute to shed light on the process. It is important to note that a number of other factors could contribute to explain the differences we reported: the microbial and ecto-parasite fauna could be different in island and continental species assemblages, and the different type of habitat we sampled in (more open and drier forest in Australia, closed canopy and humid in New Zealand, and intermediate in New Caledonia) could both influence the composition of the chemical profiles. Nevertheless, the pattern we found is consistent with the crypsis hypothesis, and even if other selective pressures are eventually demonstrated to explain some of the differences between the island and continental birds in our study, the more volatile nature of the preen wax in island birds points to a previously unrecognized factor in their vulnerability. Having evolved in environments with few or no olfactory-searching predators, many native birds in both New Zealand and New Caledonia have now become critically endangered (or driven to extinction) by introduced predatory mammals. The results of our study suggest the more volatile odours produced by native island birds, via their preen wax, may be one of the causes of their vulnerability.

Overall, my study confirms the incredible wealth of information that avian chemical signals display, and the diversity in the chemical profiles across species and life stages. One important conclusion that can be applied to the conservation of island birds is that the increased volatility of the profiles of this assemblage might be one of the contributing factors to the enhanced predation rates that native birds suffer, compared to introduced birds (Starling-Windhof et al. 2010). The lack of behavioural adaptations to introduced predators and the lack of chemical adaptation both concur in making native birds more vulnerable to predators. One of the most intriguing aspects I encountered during this thesis was individuating appropriate techniques and measures to accurately assess and quantify the variation present in chemical profiles. Most of the techniques used at present were developed in the field of community ecology, and transposed within chemical ecology, equating abundances (as count) of biological species to abundances (as concentration) of chemical species within a sample. This approach has served us well so far, but it presents a set of challenges

especially regarding repeated measures analysis and quantification of temporal variation. It is hoped that, as more researchers enter the field, due attention and effort will be put into developing dedicated techniques and expanding upon existing ones.

Another challenge posed by this field of research is that, at present, it is still not clear which, if any, is the main function of preen wax. As outlined in Chapter 1, many functions of preen wax have been hypothesized and of these, a number have found support in some species and not others. It is likely that preen wax will prove to have more than one primary function, or to have been co-opted, at some stage during avian evolution, for a secondary function, much like insects cuticular hydrocarbons, which originated as protective layer against desiccation and then acquired a communication function. As I have realized during the course of this study, until more light has been made on the primary and accessory functions of preen wax, our ability to investigate the factors (both proximate and ultimate) influencing its composition will be partial at best.

Finally, the biological relevance of the chemical findings needs to be thoroughly assessed as, even for species where the presence of a certain chemical signal has been confirmed by repeated chemical studies (e.g. sex signal in petrels), field experiments have failed to show that the animals actually recognize this signal in the natural context, or that they use it for intraspecific communication. This is true for many of the avian species whose profile has been analyzed so far, and there are many more that have never been the subject of chemo-ecological studies. As birds are the most diverse assemblage of terrestrial vertebrates, there is still a lot of work to do!

References

Amo, L. et al., 2012. Sex recognition by odour and variation in the uropygial gland secretion in starlings. *The Journal of animal ecology*, 81(3), pp.605–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22220811 [Accessed September 25, 2014].

Bonadonna, F. & Bretagnolle, V., 2002. Smelling home: a good solution for burrow-finding in nocturnal petrels? *The Journal of experimental biology*, 205, pp.2519–2523. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12124375.

Bonadonna, F. & Mardon, J., 2013. Besides Colours and Songs, Odour is the New Black of Avian Communication. In M. L. East & M. Dehnhard, eds. *Chemical Signals in Vertebrates 12*. Springer New York, pp. 325–339. Available at: http://dx.doi.org/10.1007/978-1-4614-5927-9_26.

Célérier, A. et al., 2011. Chemical kin label in seabirds. *Biology letters*, 7(6), pp.807–10. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3210654&tool=pmcentrez&rendertype=abstract

Crandall, K.A. et al., 2000. Considering evolutionary processes in conservation biology. *Trends in Ecology and Evolution*, 15(7), pp.290–295.

Haribal, M., Dhondt, A. & Rodriguez, E., 2009. Diversity in chemical compositions of preen gland secretions of tropical birds. *Biochemical Systematics and Ecology*, 37(2), pp.80–90. Available

at: http://dx.doi.org/10.1016/j.bse.2008.12.005.

Hirao, A., Aoyama, M. & Sugita, S., 2009. The role of uropygial gland on sexual behavior in domestic chicken Gallus gallus domesticus. , 80, pp.115–120.

Holderegger, R., Kamm, U. & Gugerli, F., 2006. Adaptive vs. neutral genetic diversity: implications for landscape genetics. *Landscape Ecology*, 21(6), pp.797–807. Available at: http://link.springer.com/10.1007/s10980-005-5245-9 [Accessed July 13, 2015].

Leclaire, S. et al., 2014. Preen secretions encode information on MHC similarity in certain sex-dyads in a monogamous seabird. *Scientific Reports*, 4, p.6920.

Leclaire, S. et al., 2012. Semiochemical compounds of preen secretion reflect genetic make-up in a seabird species. *Proceedings of the Royal Society B: Biological Sciences*, 279(1731), pp.1185–93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3267147&tool=pmcentrez&rendertype=abstract [Accessed February 18, 2015].

Mardon, J. et al., 2010. Species, gender, and identity: cracking petrels' sociochemical code. *Chemical senses*, 35(4), pp.309–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20190009 [Accessed January 21, 2014].

McKay, J.K. & Latta, R.G., 2002. Adaptive population divergence: Markers, QTL and traits. *Trends in Ecology and Evolution*, 17(6), pp.285–291.

Noble, R.E. et al., 1963. Incorporation of Propionic Acid Into a Branched-chain Fatty Acid of the Preen Gland of the Goose. *Nature*, 199, pp.600–601.

Reneerkens, J., 2005. Switch to diester preen waxes may reduce avian nest predation by mammalian predators using olfactory cues. *Journal of Experimental Biology*, 208(22), pp.4199–4202. Available at: http://jeb.biologists.org/cgi/doi/10.1242/jeb.01872 [Accessed March 5, 2015].

Reneerkens, J., Piersma, T. & Damsté, J.S.S., 2002. Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why? *Proceedings of the Royal Society B: Biological Sciences*, 269(May), pp.2135–2139.

Starling-Windhof, A., Massaro, M. and Briskie, J.V. 2010. Differential effects of exotic predator-control on nest success of native and introduced birds in New Zealand. *Biological Invasions* 13(4): 1021-1028. http://dx.doi.org/10.1007/s10530-010-9886-5.

Thierry A (2015) Predation risk and the evolution of odours in island birds. Doctoral Thesis, University of Canterbury, Christchurch, New Zealand.

Thomas, R.H. et al., 2010. Use of TLC-FID and GC-MS／FID to examine the effects of migratory state, diet and captivity on preen wax composition in White-throated Sparrows *Zonotrichia albicollis*. *Ibis*, 152, pp.782–792.

Tregenza, T. & Wedell, N., 2000. Genetic compatibility, mate choice and patterns of parentage: Invited review. *Molecular Ecology*, 9, pp.1013–1027.

Zelano, B. & Edwards, S. V, 2002. An Mhc Component to Kin Recognition and Mate Choice in Birds: Predictions, Progress, and Prospects. *The American Naturalist*, 160(S6), pp.S225–S237.